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Essays on Organismal Aspects of the Fungus-Growing Ant Symbiosis: Ecology, Experimental Symbiont Switches and Fitness of *Atta*, and a New Theory on the Origin of Ant Fungiculture

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Essays on Organismal Aspects of the Fungus-Growing Ant Symbiosis: Ecology, Experimental Symbiont Switches and Fitness of *Atta*, and a New Theory on the Origin of Ant Fungiculture

by

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Dedication

To my son

To Kristin

To all my family

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Essays on Organismal Aspects of the Fungus-Growing Ant Symbiosis: Ecology, Symbiont Switches, Pathology and Fitness of *Atta*, and a New Theory on the Origin of Ant Fungiculture

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This dissertation includes several experimental approaches aimed at elucidating coadaptations between leaf-cutting ants and their symbiotic fungi.

The second chapter provides ecological information on the fungus-growing, leaf-cutting ant used as study organism, *Atta mexicana* from Northeastern Mexico. A new commensalistic myrmecophile was discovered: a highly specialized moth, *Amydria anceps* (Lepidoptera: Acrolophidae), whose larvae live gregariously on the spent fungal substrate of the *A. mexicana* colony.

Although there is an intuitive conception that there is a "high" level of ant-fungus coadaptation in the higher attines (particularly in the leaf-cutters), there are no empirical data on these alleged mutual adaptations. In Chapters 3 and 4, experimental fungal symbiont switches between *Atta* (derived) and *Trachymyrmex* (basal) provide the first evidence of tangible coadaptatations and negative effects restricting switches to novel

cultivars. Striking effects were observed when *Atta* ants cultivated the *Trachymyrmex* symbiotic fungus. These include severely restricted fungus and ant colony growth, with reduced worker sizes and numbers.

In Chapter 5 the effect of fungal change on the physiology of leafcutter ants is addressed; specifically, on the mortality of workers upon challenge with the insect-pathogenic fungus *Beauveria bassiana*. I developed and compared two tetratrophic systems (chains) each using a different fungal symbiont; the energy flows in these systems were as follows: plant leaves→fungal symbionts→leafcutter ants→pathogen (*B. bassiana*).

The last chapter proposes a novel theory on the origin of the ant-fungus symbiosis. This mutualism is suggested to originate from the opportunistic consumption, by the attines' ancestor, of the fungi derived from a preexisting insect-fungus mutualistic symbiosis, such as ambrosia beetles (Coleoptera: Curculionidae) or wood wasps (Hymenoptera: Siricoidea). This subsequently led to conservation of these fungi and to their cultivation. Therefore, theories on the origin of the attine symbiosis can be separated into three groups as follows: 1) "consumption first" hypotheses that propose *de novo* domestication and cultivation of free-living fungi from different sources; 2) the "phoresy-consumption" hypotheses, which propose a system whereby fungi first utilized the ants as means of transport (phoresy) with subsequent development of consumption and cultivation; and 3) the "exploitation of preexisting symbiosis" hypothesis herein proposed for the first time.

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Chapter 1: Introduction: General biology of fungus-growing ants, the *Trachymyrmex* and *Atta* symbioses and the *Trachymyrmex* symbiosis paradox.

Fungus-growing ants (Hymenoptera: Formicidae, Attini) are a fascinating and diverse group comprising about 200 species in 13 genera. A monophyletic clade, all the Attini share the habit of cultivating mycelia of fungi (Basidiomycota: Agaricales: Agaricaceae and Pterulaceae) for food (Chapela et al.1994, Mueller et al. 1998; Munkacsi et al. 2004). The ants' survival depends on the successful cultivation and production of biomass of their symbiotic fungi, which is very likely the only food of larvae (Weber 1972; Cherrett 1989). Ants of the more basal attine genera *(Mycetarotes, Myrmicocrypta, Mycocepurus, Apterostigma, Cyphomyrmex, Mycetosoritis, Mycetophylax, Sericomyrmex,* and *Trachymyrmex*) tend to have small and inconspicuous colonies, usually of a few dozen to a few hundred workers. In contrast, individual *Atta* colonies can cultivate subterranean fungus gardens weighing almost 150 kg (Stahel 1943) and can produce about 5 kg of ants (Weber 1972; Cherrett 1989).

1.1 TAXONOMY

Attine ants have been divided into two groups (Mueller 2002): the "lower attines", which include *Myrmicocrypta, Apterostigma*, *Mycocepurus*, *Mycetarotes*, *Mycetosoritis, Cyphomyrmex*, and *Mycetophylax*, and the "higher attines". The "higher attines", a monophyletic group, was first proposed by Weber (1982) on morphological evidence. Wetterer et al (1998) confirmed the validity of this clade. It is subdivided in two groups: The *Trachymyrmex-Sericomyrmex* clade, and the leaf-cutter clade that includes the very advanced and phylogenetically distal *Acromyrmex* and *Atta*. The distinctive *Trachymyrmex-Sericomyrmex* clade and their symbiotic fungi are called the *Trachymyrmex* symbiosis (Currie et al. 2003). The *Trachymyrmex* symbiosis is paraphyletic with respect to the monophyletic leaf-cutter clade.

The fungal cultivars of the lower attines, which have small colonies, are highly diverse, often similar or identical to free-living mushrooms, and taxonomically different from the fungi of the higher attines; these cultivars are monophyletic and derived from within one group of lower attine-cultivated fungi (Mueller et al. 1998). There has been considerable switching and domestication of wild cultivars across genera of the lower attines (Mueller et al. 1998. In contrast, the fungi cultivated by the higher attines are restricted to two clades, which correspond to the two ant clades (i.e. the *Trachymyrmex* symbiosis cultivars and the leaf-cutter symbiosis cultivars). The fungi of the higher attines have never been found in a free-living state separate from the ants, despite considerable search efforts (e.g. Mueller et al. 1998). Both *Trachymyrmex* and *Sericomyrmex* cultivate fungi that are different but closely related to the *Atta-Acromyrmex* symbiotic fungi (Chapela et al. 1994, Sogin and Hinkle 1997; Currie et al. 2003).

Despite the similarities between the higher-attine cultivars, extensive sampling indicates that each higher-attine clade cultivates exclusively fungi from their respective strain clusters (Chapela et al. 1994; Bot et al. 2001; Currie et al. 2003; Silva-Pinhati et al. 2004; U. Mueller, personal communication; S. Rehner, personal communication). The strains across the leaf-cutter symbiosis are very similar and they are almost identical between the allopatric *Atta* and *Acromyrmex* species (Silva-Pinhati et al. 2004). Fungal switches between the higher attines (between the *Trachymyrmex* symbiosis and the leaf-cutting ants symbiosis) have never been detected in nature.

1.2 ECOLOGY

As most ants, attines carry on mating flights. In the genus *Atta* (and probably in most, if not all attines), foundress queens collect a piece of fungus from their maternal nest before they take off for the mating flight. In this way they have a “starter” inoculum to initiate their fungus garden, after they found their own new colony (Weber 1972). However, lateral (horizontal) acquisition of selected fungal cultivars, after catastrophic loss of the colonies’ own cultivar, is widespread in most attine groups (Mueller et al. 1998; Mueller et al. 2004).

The ecological roles of fungus-growing ants fall into two broad categories or guilds. Ants of nine attine genera (all the lower attines: *Apterostigma, Myrmicocrypta, Mycocepurus, Mycetarotes, Cyphomyrmex, Mycetosoritis, Mycetophylax*, plus the higher attines *Sericomyrmex* and *Trachymyrme*x) tend to have small and inconspicuous colonies (usually much less than 2000 and as few as 20 workers in mature colonies). These scavenger or detritovore genera utilize small pieces of dead plant material, like fallen fruits, flowers and inflorescences (e.g. catkins), insect corpses, wood fragments, insect feces (from caterpillars, wood beetles, and grasshoppers), fragments of fruiting bodies of fungi, and plant debris in general. Thus, these ants share a broad guild with organisms such as the Collembola, Diplopoda, terrestrial isopods, and oribatid mites as decomposers of plant material and organic debris (via the symbiotic fungus in the case of attines) (Hopkin 1997, Garcia et al. 2000, Hubert et al. 2000, Kaspari 2001). These higher- and lower-attine detritovores may even share parasites (such as the mite *Garmania* and the garden robber ant *Megalomyrmex*), which are unknown among leaf-cutter ants (Weber 1972) thus emphasizing their similarity.

In contrast, ants belonging to the highly differentiated genera *Acromyrmex* and *Att*a have colonies that can grow to include tens of thousands to several million workers. These two genera of ants depend on cutting fresh leaves and vegetation for their fungal gardens and consequently are commonly called ‘‘leaf-cutting’’ ants (Wetterer et al. 1998). Many species belonging to these two genera are major agricultural pests in the Western hemisphere. Functionally, the leaf-cutter ants *Acromyrmex* and *Atta* are extremely polyphagous herbivores and thus seem to compete with herbivores like howler monkeys and sloths (Rockwood and Glander 1979; Estrada and Coates-Estrada 1986).

An apparent inconsistency arises when comparing fungal-ant phylogenies, symbioses, and ecologies. The higher attine genera *Trachymyrmex* and *Sericomyrmex* are ecologically and functionally more like the primitive attines yet they cultivate derived fungi very similar to those of the leaf-cutters (Chapela et al. 1994; Wetterer et al. 1998). Both lower- and higher-attine detritivores are small, often of comparable individual and colony sizes, and they are monomorphic (having uniform worker size within colonies).

The very primitive *Myrmicocrypta* have colonies as large or larger (in volume, fungal biomass and worker numbers) than those of *Sericomyrmex* and *Trachymyrmex* species (Weber 1972). Worker numbers in colonies of *Trachymyrmex* genus match those of some *Cyphomyrmex* species, a more basal genus (J. Longino, personal communication). In terms of ant and fungal biomass production, the difference between the detritovore higher attines and leaf-cutting colonies is immense. Thus we have small, inconspicuous, monomorphic ants (*Trachymyrmex* and *Sericomyrmex*) cultivating very derived fungi. These ants presumably have low nutritional requirements. Thus, it is hard to propose that similar selective pressures exerted on these genera resulted in the maintenance of not identical but very similar, very derived fungi in both *Trachymyrmex* and *Atta*, for example. Why have these small and inconspicuous ants adopted rather advanced cultivars? Have they "capitalized" on strains that were improved (nutritionally?) while associated with other ant lineages (leaf-cutters), as suggested by Mueller et al (1998)? Villesen et al. (1999, 2002) had previously pointed out the transitional nature of *Trachymyrmex* and *Sericomyrmex* in the general attine phylogeny.

In my work, I attempted to clarify experimentally coadaptations limiting symbiotic fungus switches between the *Trachymyrmex* and leaf-cutter symbioses. I also developed a new hypothesis on the origin of the fungus-growing ant symbiosis, and gathered ecological data on the little-investigated Mexican leaf-cutting ant, *Atta mexicana* (Smith).

Chapter 2: Ecological Observations on *Atta* in Northeastern México: Distribution, Mating Flights, Female Behavior, Commensals and Natural Enemies.

Synopsis: *Atta mexicana* females were collected in the Monterrey, Nuevo León area, in Northeastern (NE) México for the experimental purposes of this dissertation. Very little information exists on *Atta* species in this part of México; thus, data on colony distribution, mating flights, pre- and post-flight behavioral changes of females, and commensals and natural enemies are reported for *A. mexicana* collected in 2000-2002 in the Mexican states of Tamaulipas and Nuevo León.

2.1 INTRODUCTION

In Northeast (NE) México (states of Tamaulipas and Nuevo León) the distribution of *Atta mexicana* (Smith) and of *Atta texana* (Buckley), are reported to come within 100 miles or less of each other. These two *Atta* species are putative sister species of leaf-cutting ants, the two northernmost members of this genus in the Americas. In the NE México-Texas area, the geographic distribution records of each species indicate either an allopatric distribution, or a narrowly sympatric one. For example, Smith (1963) and Moser (1967) report *A. texana* occurring along the coast of the Gulf of México south to Veracruz, where it would be sympatric with *A. mexicana* and possibly also with *Atta cephalotes* (L.). There are no known records of *A. mexicana* in Texas, although this species is present in the USA within a few miles of the México border in the Organ Pipe Cactus National Monument, Arizona (Smith 1963).

There is a paucity of biological information for *A. mexicana*. I report here information on the distribution, mating flights, female behavior, commensals and natural enemies of *A. mexicana* in NE México.

2.2 DISTRIBUTION

Collecting. In July-August 2000, *A. mexicana* workers were collected in the Mexican states of Nuevo León and Tamaulipas, which border Texas to the South (Fig. 2.1). I conducted directed searches in this extensive area; I also asked local people about the presence of these conspicuous ants. Ethanol-preserved workers (majors and/or soldiers) were deposited at the insect collection of the Mueller laboratory at the University of Texas at Austin. No material was collected in NE México that matched the descriptions of *A. texana*, and therefore the reports of Moser (1967) and Smith (1963) could not be confirmed.

In general, *A. mexicana* was widespread between Pesquería and Monterrey, in Nuevo León; between Monterrey and Ciudad Victoria, Tamaulipas; in Tamaulipas, between Ciudad Victoria and Soto La Marina, and between Soto La Marina and San Fernando (Figure 2.1). Along the Gulf of México coast of Tamaulipas, and traveling on a South-North direction, the distribution of *A. mexicana* appeared to be continuous all the way to the city of San Fernando, where it was observed about 100 km south of the Texas border. The northernmost points where I observed thriving mature colonies of *A. mexicana* in NE Mexican states were (by state): in Coahuila: Sabinas; in Nuevo León: Sabinas Hidalgo; in Tamaulipas: San Fernando (Fig. 2.1).

Pest control professionals (n =10) were asked about the presence of *A. mexicana* in the cities of Matamoros, Valle Hermoso and Reynosa, in the aforementioned agricultural plain North of San Fernando, Tamaulipas. They all reported the absence of this conspicuous insect pest. A farmer, about 60-years old, living in the vicinity of Matamoros (Ejido El Galaneño) described both the absence of the insect in Matamoros and its nearest occurrence to the south, in San Fernando. In the latter city the insect is widespread, and the insecticide product Patron ™ (sulfluramid bait; FMC Corp.) is specifically marketed for *Atta* control and is widely available. In the state of Nuevo León, local farmers mentioned the absence of the insect in the towns of China and General Bravo, in Nuevo

León, on the San Juan River, about 50 km SW from Reynosa and 100 km to the east of Monterrey where the insect is abundant.

Human land use, soil factors and climate (Figure 2.1) are the most likely factors that determine the distribution of *Atta*. North from San Fernando, a continuous plain of very intensive agriculture extends to the Rio Grande. Annual crops (corn and sorghum) are cultivated there. This particular agricultural region generally is unfavorable for the establishment of *A. mexicana* for the following reasons: (1) its predominantly clayey soils (vertisols) are prone to flooding, have very poor drainage and aeration when wet and are extremely heavy and lack rocky outcrops; (2) the extensive use of agricultural pesticides; (3) an almost complete lack of native vegetation cover and of refuges; (4) and the scarcity of suitable forage plants. Wetterer et al. (1998) attributed the absence of *Atta* species at Palo Verde, Costa Rica likewise to the poor drainage and aeration of the local vertisols. In NE México, *Atta* spp. are also apparently absent from the climatic zone "Subtropical Arid Hot" (Figure 2.1), the hottest and driest climate in this region (INEGI 1983; INIFAP 2004); however, in this zone, climate is possibly more important than soil use, soil type and vegetation as limiting factor preventing establishment of colonies of *A. mexicana* and *A. texana*.

I collected *A. mexicana* near the springs ("ojos de agua") at the towns of Pesquería and Sabinas Hidalgo, Nuevo León, both of which are about 100 km straight south from the Texas border. Additional collecting indicated that at the boundary of its NE range, *A. mexicana* distribution extends south of San Fernando, in Tamaulipas, and of Pesquería, Nuevo León, and Sabinas Hidalgo, Nuevo León and possibly south of a line connecting these points.

2.3 MATING FLIGHTS IN THE MONTERREY, NUEVO LEÓN AREA

In the Monterrey area, mating flights of *A. mexicana* were observed on 2 July 2001, 4 July 2002, and 16 August 2004. In general, the mating flights closely resembled the descriptions for the mating flights of *A. texana* in Louisiana (Moser 1967). Moser et al (1998) report that individual colonies of *A. texana* commonly have multiple flights in the

same year. However, for individual nests of *A. mexicana* in Monterrey, mating flights apparently occur only one night per year, based upon the observation of nest "preparation": clearing of vegetation on and above mounds, and construction of large exit holes (about an inch or more in diameter) that are thatched with sticks and plant debris prior to flights (Moser 1967). Flights take place right before sunrise, as with *A. texana*, where mating flights "last less than 15 minutes and terminate just before the first traces of sunlight appear in the sky" (Moser et al 1998). In Monterrey, these nocturnal flights took place after heavy thunderstorms; in both 2001 and 2002 they took place the night after the heaviest precipitation of the year until that date.

I observed alate ants (sexuals) from a total of seven nests. The flight patterns were similar on both dates. However, in 2002 the street lights (light poles) near two nests (closest one at 30 meters) were turned off at one observation location. There, the ants emerged by 1 AM, while at the same location when light was present on 2001, sexuals did not emerge until 3 AM. Sexuals of *A. mexicana* are photophobic; if illuminated directly with flashlights at or near the nest entrances, they rush back into the nest. Under streetlights, males emerged first from three nests out of seven; in dark localities, females appeared before males in three nests out of seven. I couldn't witness which sex emerged first on the remaining nests. At one nest workers and alates (sexuals) were on the surface by midnight. This is earlier than the reports of Moser (1967) and Moser et al. (1998) for *A. texana*, where workers and alates first appear about 4 hours (between 1 and 2 AM) before takeoff, which happened around 5:30 AM depending on the time of sunrise.

After sexuals congregated on the nest's mounds they milled about the mound for several hours and until the actual flight took place right before dawn, when the first daylight was visible at about 5:45 AM, as described for *A. texana* by Moser (1967). At sunrise, thousands of winged sexuals littered the ground next to a business with large fluorescent lights. These ants had hit on lamps and adjacent walls and windows violently and many appeared injured.

2.4 FEMALE BEHAVIOR CHANGES AFTER MATING FLIGHTS

Virgin winged females were collected from the surface of nests in San Pedro, Nuevo León, immediately before flying on 4 July 2002. These females (n=10) were placed together in one plastic container (15 cm across), which was covered with moist synthetic cloth, provided with a piece of *A. mexicana* fungal cultivar from Monterrey and their survival and behavior were observed in the laboratory.

These 10 females coexisted peacefully for at least one month in the container. If housed together under similar circumstances, wingless (presumably mated) females of *A.mexicana* collected right after the mating flight fight ferociously, clipping each other's antennae and legs (Mintzer 1990; personal observations). Interestingly, mated females of *A. texana* coexist peacefully after the mating flights (Mintzer 1990). Unfortunately no parallel observations could be carried out to differentiate the behavior of winged (presumably unmated) and wingless (presumably mated) females *after* the mating flight to detect differences between these two groups. However, these observations indicate a remarkable change in female behavior (aggression) in *A. mexicana* within a span of an hour or less, right before and right after the mating flight. Similar observations (female collection and laboratory confinement) were carried out on 32 virgin females in 16 August 2004, with identical results.

2.5 COMMENSALS, PREDATORS AND PATHOGENS OF *A. mexicana* IN NORTHEASTERN MÉXICO

2.5.1 *Amydria anceps* Walshingham (Lepidoptera: Acrolophidae).

The hitherto unknown myrmecophilous lifestyle of a novel commensalistic, gregarious, and extremely specialized lepidopteran was described in Sánchez-Pena et al. (2002). This publication includes photos and drawings of the habitat, adults, larvae, larval tubes, and genitalia. A brief description follows.

After harvesting the fungus that it cultivates for food, *A. mexicana* eliminates the exhausted, compost-like, fungal substrate by dumping it on a garbage heap outside the nest (Figure 2.2). The exhausted fungal substrate is made up of particles or granules up to a few mm. in diameter. These particles accumulate in external mounds that can reach a volume of one cubic meter or more.

In August and September 2000, a specialized, colonial caterpillar, *Amydria anceps* (Walsingham) (Lepidoptera: Acrolophidae) was discovered that feeds on and completes its life cycle in these external dumps, which consist mainly of fungal cell walls in particles.

The caterpillars burrow into the fungal substrate heap. These larvae spin a tough, leathery tube covered with fungal substrate particles. They apparently live in these tubes through the larval cycle, protruding only the head outside the tube to feed while immersed in the substrate. Two adult emergence events were observed during the summers of 2000 and 2001, within two days after heavy precipitation. Adult emergence takes place apparently triggered by rain. The pupae take on a peculiar position right before adult emergence; they stick out from the tube mouth, at the heap surface, where pupal cuticles (sometimes hundreds) can be observed nearly perpendicular to the substrate surface, looking like a tiny army (Figure 2.4). The physical details of adult emergence are unclear and it remains unknown whether it is the pupae or the adults that wiggle to the outside leaving the pupal cuticle behind.

Walter et al. (1938) reported a morphologically similar lepidopteran, *Psilopsaltis* (=*Amydria*) *confusella* (Dietz) (Lepidoptera: Acrolophidae) from subterranean dump chambers where the Texas leaf-cutting ant, *A. texana*, accumulates the spent fungal substrate. This is, to our knowledge, the only report of a similar organism. They described larvae and adults from these subterranean waste chambers. The utilization of the subterranean chambers of *A. texana*, probably requires adaptations, behaviors and cues different from those required to exploit the exposed heaps of *A. mexicana*. No additional details are known of the life histories and adaptations of these lepidopterans.

Field-collected larvae were reared to adulthood on food of spent fungal substrate in 1-liter containers. Parasitic wasps (Hymenoptera: Ichneumonoidea) and flies (Diptera: Tachinidae) emerged from some of these reared larvae. The substrate contained no other potential insect hosts of these obligate parasites. These parasites, as well as chalcid wasps (Hymenoptera: Chalcididae), were observed engaged in apparent host searching behavior on dumps heavily colonized by larvae. The chalcids could be primary parasitoids or hyperparasitoids. The potential trophic levels of this system are thus five (plant material--symbiotic fungus--*Amydria* --ichneumonids and tachinids--chalcids).

2.5.2 *Attaphila* sp. (Blattaria: Attaphilidae).

The genus *Attaphila* includes the worldwide smallest-known roaches (order Blattaria). They are obligatory commensals of *Atta* spp. in the USA and South America (Moser 1964). Individuals were observed at several points in the urban area of Monterrey, in the Mexican state of Nuevo León. To my knowledge this is the first report of this myrmecophilous roach genus from México.

Individuals of *Attaphila* were collected riding on the body of virgin winged females aggregated outside the entrances of nests, and getting ready to take off for the nuptial flight. In 2002, ten *A. mexicana* females yielded two roaches; in 2004, 32 females yielded two roaches. It is not known if single females could carry two roaches. In the laboratory, roaches positioned themselves under the petiole of females. Roaches and female *Atta* ants were collected at 2 AM, 4 July 2002, as the sexuals congregated on the nest mounds (the actual flight taking place at dawn as mentioned), in the city of San Pedro Garza Garcia, Nuevo León, within the urban area of Monterrey.

That same day, at 8 AM, individuals of *Attaphila* were observed running on the ground, at least 30 meters from nest mounds, after the mating flight had taken place. They look like tiny beetles. In this location, the *Atta* females had fallen to the ground and they were trying to find cracks on a concrete structure bordering the surface where they landed, so roaches and ants were walking along the concrete base, being unable to climb it. The

roaches seemed to follow on the tracks of the females, as reported by Moser for *Attaphila fungicola* Wheeler (1964).

Four *Attaphila* individuals of unknown sex were separately confined on plaster nests along with one *A. mexicana* dealated female. The ant females engaged in colony founding activities [expulsion of a fungal pellet from its infrabuccal pocket, manuring and cultivation of the fungus, oviposition and larval breeding (Weber 1972)]. The roaches survived a maximum of 15 days. In this time period they were not observed to feed on the fungus or eggs at all. They sometimes followed the females and tried to climb onto them, or positioned themselves on the nest floor under the ants' petiole. They rode and licked the females eagerly when allowed. *Attaphila* has been reported to lick and feed on body secretions of their leaf-cutter ant hosts (Moser 1964; Hölldobler and Wilson 1990). Females reacted aggressively and sometimes attempted to bite the *Attaphila.* Thus, the roach did not seem to be accepted completely by its female host.

2.5.3 *Beauveria bassiana* (Bals.) Vuill. and a *Cordyceps*-like Pathogen.

A total of three females from among 250 (1.2%) collected right before and after the 2002 mating flight developed fungal infections. Fungi were identified from the descriptions in Samson et al. (1988). The mitosporic (asexual) fungus *Beauveria bassiana* (Deuteromycotina: Hyphomycetes: Moniliales) grew out of a virgin, winged female collected before the mating flight. This fungus also infected a wingless female collected after the mating flight. Typical *B. bassiana* growth emerged from inside the females, eventually covering the carcasses (Fig 2.2A).

Another winged female collected after the flight died of fungal infection within a week as well. The fungus emerged and produced many yellowish-whitish, thin, long fruiting bodies (approx. 3 mm thick and up to 10 cm long), bearing the asexual (*Beauveria*) conidiogenous structures. Such conidia-producing fruiting bodies are termed synnema (pl. synemmata) (Figure 2.2B). This fungus belongs in all probability in the Clavicipitaceae (Euascomycotina: Clavicipitales): it is a *Beauveria* species growing in a *Cordyceps*-like form. Several *Cordyceps* species, like *C. scarabaeicola* Kobayasi &

Shimizu have asexual (conidiogenous) phases that correspond to *Beauveria* and allied genera (Samson et al. 1988; Sung 1996).

Natural infections by *Beauveria bassiana* on leaf-cutting ants have been reported several times (Sánchez-Peña 1990; Diehl-Flieg et al. 1992). Another Deuteromycotina, *Metarhizium anisopliae* (Metsch.) Sorokin also infects *Atta sexdens rubropilosa* Forel and *Atta bisphaerica* Forel (Jaccoud et al. 1999). Chapter 5 provides an overview of the fungal entomopathogens of leaf-cutting ants.

2.5.4 *Nomamyrmex esenbecki* (Westwood) (Hymenoptera: Formicidae: Ecitoninae).

A nocturnal raid of the army ant, *Nomamyrmex eisenbecki* (Westwood) preying on *A. mexicana* brood was observed in the municipality of Soto La Marina, in the Mexican state of Tamaulipas. This raid was described in Sánchez-Peña and Mueller (2002). The full report follows:

Neotropical army ants (Hymenoptera: Formicidae, Ecitoninae), as a group, are primarily predators of the immature stages of ants, termites and some wasps (Rettenmeyer 1963; Schneirla 1971). Different species of army ants have marked preferences for attacking specific ant taxa (subfamilies or genera) (Rettenmeyer et al.1982; Franks and Bossert 1983; Franks and Norris 1987), displaying variable prey preference among all major subfamilies of ants with the exception of the Ecitoninae itself (Rettenmeyer 1963; Schneirla 1971; Rettenmeyer et al.1982; Gotwald 1995). However, predation by army ants on the often massive colonies of leaf-cutting ants, *Atta* and *Acromyrmex* (Hymenoptera: Formicidae, Myrmicinae, Attini) has very rarely been reported. *Atta* spp. and army ants in the genus *Eciton* usually avoid confrontation and ignore each other (Rettenmeyer 1963); *Neivamyrmex* army ants have even been reported as inquilines in the nest cavities of *Atta* (Schneirla 1971). On the other hand, the few observations on foraging by the uncommon, robust, heavily sclerotized ecitonine *Nomamyrmex esenbecki* suggest that these army ants seem to be rather specialized predators of the brood of species of *Atta* and *Acromyrmex*, and particularly of *Atta* spp. (Swartz 1998). All reports

of prey of *N. esenbecki* mention leaf-cutting ants, and it appears that all reports of army ant raids on *Atta* and *Acromyrmex* nests involve *N. esenbecki* (Borgmeier 1955; Mariconi 1970; Rettenmeyer 1963; Rettenmeyer et al.1982; Swartz 1998, and references therein; J. Longino, personal communication). In Costa Rica, *N. esenbecki* is the only army ant observed to attack mature *Atta* colonies (J. Longino, personal communication). 80-90% of *N. esenbecki* diet consists of ant larvae and pupae (Rettenmeyer 1963). Working in the Panamanian rainforest of Barro Colorado, Schneirla (1971) reported that *N. esenbecki* is a subterranean species that is also capable of surface activity and often raids in dense forests both day and night.

Here I describe a new distribution record for *N. esenbecki*, and for its raids on *Atta*: namely, a nocturnal surface raid on an *A. mexicana* colony in NE México. This army ant raid took place at the northeastern fringe of the Neotropical zone, extending the known occurrence of *Nomamyrmex* raids on leaf-cutting ants more than 1000 km to the north of Jalisco, México. It occurred in a disturbed subtropical habitat, on a clearing inside a village. Most reports of *Nomamyrmex* surface raids against *Atta* are from tropical rain forests in Central and South America. The previous northernmost raid observed, in a subtropical area (Jalisco), was subterranean (Rettenmeyer et al.1982).

The locality of the raid was the village of Buenavista, in the municipality of Soto La Marina, Tamaulipas, México (27º47' northern latitude; 90º12' western longitude; 20 meters altitude above sea level), about 200 km south of the United States border.The climate, in the classification of Koppen and modified by García (INEGI 1983; INIFAP 2004) is BS (h') KW (e): subtropical semiarid hot, extremely variable, mean annual temperature 23º C; annual precipitation: 800-1000 mm, mainly in the summer. Natural vegetation is disturbed in the village; native plants reflect the boundary of low tropical thorn forest and low deciduous tropical forest; native trees and shrubs are ebony (*Phitecellobium flexicaule* (Benth.) Coult), cornezuelo (*Acacia cornigera* (L.) Willd.), huisache (*Acacia farnesiana* (L.) Willd.), brasil (*Condalia sp.*), coma (*Bumelia* sp.), and *Randia* spp.

The raid occurred in a clearing (lawn) of introduced blue grass, *Poa pratensis* L., on sandy soil. The *Atta* colony was under a small orange tree (canopy diameter. 2.5 m) at least 20 m from other significant tree or bush cover and inside a farmer's garden located in the village. The colony was small (mound less than 1 m^2) and had two entrance holes on the mound. From the mound and worker size, this *Atta* nest was probably about 2 years old. *Nomamyrmex* workers were carrying *Atta* larvae and possibly pupae out from this small *A. mexicana* colony. A column of polymorphic *Nomamyrmex* workers (up to 11 mm long) running in a single line, were exiting one of the nest holes of the *Atta* mound at 21:00 h on 15 June 2000. They were very swift runners; this made their capture at night difficult. The army ants were very photophobic and when illuminated by a flashlight within a meter from an entrance hole, immediately retreated back into the *Atta* nest, and exiting from the hole was interrupted. About twenty to thirty seconds after turning the light off, the ants resumed their normal activity and left the exit hole. Approximately one in every ten *Nomamyrmex* workers carried one ant larva each, which were later identified as *A. mexicana* by Dr. Ted Schultz, Smithsonian Institution; the *Nomamyrmex* could have been carrying their own larvae besides *Atta.* No aggressive behavior was observed from the few *Atta* workers present on the mound; these workers stood still or slowly walked around the nest entrances. Only minors and media *Atta* workers (no majors or soldiers) were observed. The exodus of *Nomamyrmex* from the *Atta* nest continued for at least 3 h, until midnight (24:00 h) when observation was suspended. The army ants left the *Atta* nest at a steady rate of no less than one worker every two seconds; therefore this *Nomamyrmex* colony had a minimum of 5400 workers**.** Rettenmeyer (1963) described similar swift column raids.

Historically, *N. esenbecki* has been collected in the USA, from "southern Texas" (Rettenmeyer 1963), including Cameron county in the Lower Rio Grande Valley (LRGV), extreme south Texas; *Atta texana* has been reported from adjacent counties in the Valley (O'Keefe *et al.* 2000). Currently, most of the LRGV supports very intensive agriculture and pesticide use (Howe et al. 1986). *Atta texana* has pest status there and possibly forages almost exclusively in human-disturbed areas, since native vegetation

cover in the LRGV has disappeared in more than 95% (Howe et al.1986, TAMU 2001). Mature *Nomamyrmex* colonies are huge (Swartz 1998); estimates are > 700,000 workers (Rettenmeyer 1963). Such species of army ants are unable to survive in extensively disturbed areas (Swartz 1998). The recent association of *Atta* with man and the extirpation of natural habitats in the LRGV could imply that *Nomamyrmex* is being eliminated from the United States, if not already extinct there.

Like most *Atta* species of the Neotropical region, *A. mexicana* colonies are common in both natural and disturbed areas (Hölldobler and Wilson 1990), including in Tamaulipas where this raid occurred. Topics deserving further study are the adaptation and current status of *Nomamyrmex* in disturbed, subtropical areas, and more specifically its biology at the northern edge of its distribution, the impact of army ants as a mortality factor of young and mature *Atta* colonies, and the architectural, chemical and behavioral defenses of *Atta* ants against raids by these army ants.

Figure 2.1. Climate map and general distribution of *Atta mexicana* and *Atta texana* in the Northeastern Mexican states of Coahuila, Nuevo León and Tamaulipas.

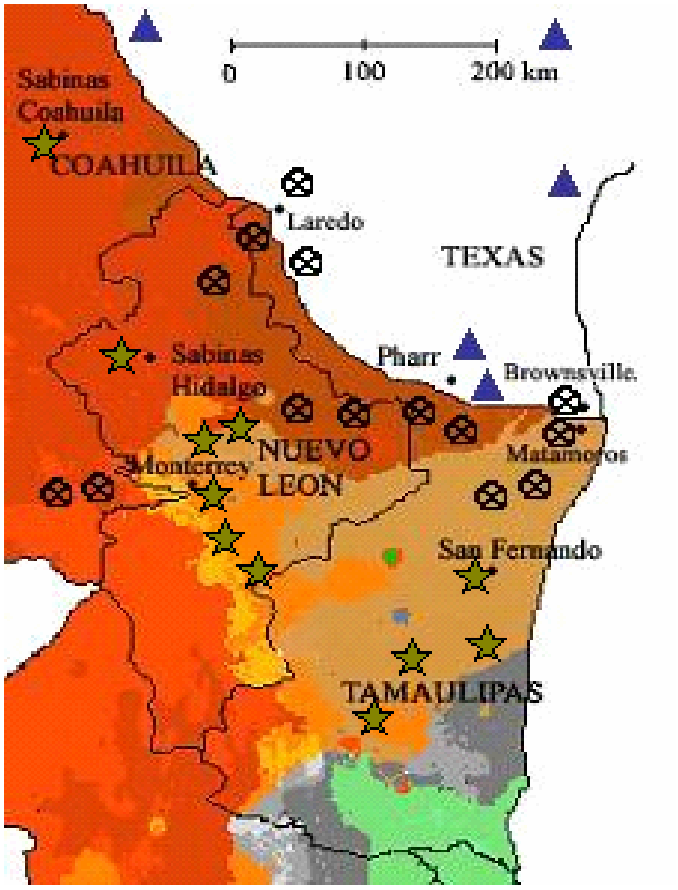


Figure 2.1 continued. Selected Texas points are shown; see O'Keefe et al. (2000) for distribution map and county list of *Atta texana* records in Texas.
Legend: ★ = *A. mexicana* colonies; ▲ = *A. texana* colonies observed in this work and/or reported by O'Keefe et al. (2000) (selected Texas points shown herein); ⊗ = No *Atta* spp. colonies observed, and insect reported not to exist in such localities by residents and/or local pest control operators.

The northernmost points where I observed thriving mature colonies of *A. mexicana* in Mexican states are indicated in the map. They are the following (by state): in Coahuila: Sabinas Coahuila; in Nuevo León: Sabinas Hidalgo; in Tamaulipas: San Fernando.

The climate Subtropical Arid Hot (roughly between Laredo and Brownsville, on the Mexican side) is possibly unfavorable to the establishment of *Atta* colonies. This might explain the absence of *Atta* spp. from this climatic zone.

Map courtesy of INIFAP (Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias), México (INIFAP 2004)

Figure 2.2. *Amydria anceps* colonial larval tubes. Tubes are made of silk and fungal refuse particles, from the garbage dumps of *Atta mexicana*. Monterrey, Nuevo León, Mexico.

Figure 2.3. *Amydria anceps* pupal cases on *Atta mexicana* fungal dump. Cases empty shortly after massive emergence. Monterrey, Nuevo León, Mexico.

Figure 2.4A and 2.4B. Natural fungal infections of *Atta mexicana* queens. Monterrey, Nuevo León, México.

2.4A. A *Beauveria bassiana*-killed *A. mexicana* queen. Head is to the left. Notice fungal emergence through softer cuticle at leg joints.

2.4A.

Figure 2.4B continued.

Cordyceps-like synemmatous form of *Beauveria* emerging from winged *Atta mexicana* female. Notice the tubular and clavate fruiting bodies (synemmata) and the rhizoid-like growth (rhizomorphs) attaching both ant and fungus firmly to the substrate. There is a piece of yellow sponge for moisture.

2.4B.

Chapter 3: Some Growth Parameters of Fungal Gardens in Native and Novel Symbiont Pairings in Mini-nests

Synopsis: Loss, contamination, or mixing of fungus gardens are thought to be possible mechanisms leading to fungal symbiont replacement in the fungus-growing ants (Mueller et al. 1998; Green et al. 2002; Mueller 2002). Unlike the lower attines, which ostensibly change fungal cultivars within ecological time spans, fungal switches in the higher attines (between members of the *Trachymyrmex* symbiosis and the leaf-cutter symbiosis, *sensu* Currie et al. 2003) have never been detected in nature (Chapela et al. 1994; Mueller et al. 1998; Bot et al. 2001; Silva-Pinhati 2004; U. G. Mueller, personal communication; S. Rehner, personal communication). This chapter aims at elucidating specific functional mismatches between novel fungal and ant symbionts across the higher attines. Thus, in order to identify some of the constraints against symbiont changes, experimental symbiont switches were established in mini-nests, focusing mainly on fungus-growing ants of the genus *Atta* experimentally switched to cultivate a fungus from *Trachymyrmex zeteki*. Mini-nests are nest boxes with a fragment of fungal symbiont garden, plus workers only; no fertile queens are present. Experimental manipulations involving brood, nestmates and fungal symbionts were central in these observations: newly emerged adult (callow) workers of *Atta* were provided with either native or novel (*Trachymyrmex*) cultivars, which the workers accepted and cultivated. When cultivated by *Atta* workers, the *Trachymyrmex* fungus symbiont grew less in biomass, and produced on average fewer nutrient cells (gongylidia) compared to the native (*Atta*) symbiont. If these findings can be generalized, it seems thus disadvantageous for an *Atta* colony to adopt and cultivate *Trachymyrmex* fungal symbionts. These observations implicate specific coadaptations that have evolved between the partners in the *Atta* symbiosis, which might exclude switching to fungal symbionts outside the leafcutter-fungus clade.

3.1 INTRODUCTION

There is an extensive body of literature on recognition of self, brood, kin, caste and colony in ants (Crozier and Dix 1979; Hölldobler and Wilson 1990; Vander Meer and Morel 1998; Vander Meer and Alonso 2002). In general, "learning" of a very restricted and predictable nature has been implicated in colony member discrimination (Hölldobler and Wilson 1990). In attine ants, colony recognition is further complicated by the inclusion of the fungal symbiont in the nest. Very precise (and possibly essential) identification mechanisms restrict the number of cultivars grown by many attines to limited clusters of extremely similar strains (Mueller et al. 1998; Mueller et al. 2002). In many of these ants, the fungal strains can possibly be considered biotypes of a handful of fungal species, all closely related to *Leucoagaricus gongylophorus* (Möller) Heim (Weber 1972; Bononi et al. 1981; Singer 1986; Muchovej et al. 1991; Fisher et al. 1994a, 1994b; Pagnocca et al. 2001; Mueller 2002).

There are very few reports on recognition and acceptance of alien (herein called novel) versus their own symbiosis' (herein called native) fungal cultivars in fungus-growing ants. In nature, intraspecific, between-nest transfer of fungal cultivars by means of raiding of colonies has been observed in the leaf-cutting ants *Atta sexdens rubropilosa* (L.) (Autuori 1950) and *Acromyrmex versicolor* (Pergande) (Rissing et al. 1989). An intergeneric switch of a fungal symbionts is documented for *Apterostigma auriculatum* Wheeler and *Cyphomyrmex longiscapus* Weber (Mueller et al. 1998). Within the higher attines, one colony of *Trachymyrmex papulatus* Santschi has been found cultivating a lower attine fungus (Mueller et al. 1998) indicating a recent switch from higher- to lower attine fungus after the origin of the species *T. papulatus*. In laboratory studies, Weber (1972) comment briefly that colonies of *Trachymyrmex urichi* Forel and *Trachymyrmex septentrionalis* "repeatedly accept a fungus garden from an *Atta cephalotes* colony. The ants then proceed to care for the fragments and build up a viable garden." Weber (1972) also described other switches in the laboratory where a colony of *Acromyrmex lobicornis* Emery adopted an *A. cephalotes* fungus after garden loss, and *Acromyrmex landolti* Forel adopted an *Acromyrmex octospinosus* Reich fungus. Bot et al. (2002) describes

variability of short-term acceptance of cultivars in experimental intra- and interspecific switches among very closely related attines: workers of the sister species *Ac. octospinosus* and *Acromyrmex echinatior* Forel. It is difficult to asses the selective pressures resulting on the overall acceptance and rejection patterns reported in Bot et al (2001), since both ants and cultivars tested are respectively closely related and similar. The lower attines in general are versatile domesticators of different cultivars (Mueller et al. 1998), but sudden changes of symbiotic fungus can be a difficult process even in the laboratory. In a study of cultivar transfer in inter- and intraspecific pairings of colonies of the sister species *Cyphomyrmex muelleri* Schulz and Solomon and *C. longiscapus* (when *C. muelleri* was deprived of fungus garden) only 25% of interspecific pairings resulted in cultivar transfer, versus 100 and 78% in intraspecific pairings (Adams et al. 2000). These authors reported also that the respective fungi of these ants are distantly related and that *C. longiscapus* does not readily accept gardens of *C. muelleri*. Similarly, mature *C. muelleri* workers rejected some (but not all) different, novel cultivars from other species and genera of lower attines. In choice experiments between alien (novel) cultivars based upon their phylogenetic closeness to the *C. muelleri* native cultivar, workers generally selected the nearest relative of their native symbiotic fungus over more distantly related cultivars (Mueller et al. 2004).

My unpublished observations indicate that *Atta* spp. workers exposed to their native fungal symbiont from the time of their emergence as adults will neither accept nor cultivate a *Trachymyrmex* cultivar. Rather, they discard it, and eventually starve to death. The ants will meticulously chew the fungus (Weber 1972; Sánchez-Peña personal observations); however, it is unknown whether this chewing constitutes actual feeding, or an effective method to grind and kill undesirable fungi by crushing and bursting their cells. Similarly, mature workers from *Atta* spp. colonies will not readily accept and cultivate a fungus from other conspecific or heterospecific *Atta* colonies (although occasionally they will readily accept it). However, my unpublished observations indicate that "naïve" workers will accept such alien cultivars shortly after their eclosion as adults. Naïve workers are callow workers that after emerging as adults have never been exposed

to a fungus cultivar, nor to queen pheromones (i.e., those in a queen-right colony [colonies having a queen are called queen-right]). Thus, in my preliminary investigations, I was able to induce naïve *Atta* spp. workers to accept cultivars from *Trachymyrmex zeteki* Weber, *Ac. octospinosus*, and other, non-sympatric *Atta* species. Naïve *Trachymyrmex zeteki* workers, conversely, could be induced to accept and cultivate permanently the tested *A. cephalotes* cultivar.

The overall aim of the research discussed in this chapter was to quantify precisely the interactions between attine ants and fungal cultivars from either their own symbiosis (native) or from other attine genera (novel). I compared fungus garden growth rates and number of gongylidia produced as indicators of possible mismatches**.** In the higher attines, the ants feed their larvae with specialized clusters of swollen fungal cells produced on the surface of the fungus garden (Weber 1972). These clusters (staphylae) measure approximately 0.5-1 mm in diameter. They are formed by groups of terminally (or less frequently intercalary) swollen hyphae (gongylidia, singular = gongylidium). The individual swollen hyphae measure 20-40 microns in diameter (Weber 1972; Angeli-Papa and Eymé 1985). Gongylidia, as opposed to undifferentiated hyphae, are believed to be essential for the nutrition of larvae of higher attines in general (Weber 1972; Angeli-Papa and Eymé 1985).

3.2 MATERIALS AND METHODS

3.2.1 Fungal Garden Weight Change.

I monitored weight changes in incipient fungal gardens in mini-nests harboring different ant-fungus combinations, as follows:

Establishment of "nursing" mini-nests to obtain naïve workers. Mini-nests consisted of worker ants housed in nest boxes. Nest boxes were clear rigid plastic boxes with removable covers. Boxes were either 7 x 7 x 3 cm, or 11 x 11 x 3.5 cm (Figure 3.1). High humidity inside the nest boxes was maintained by means of either a bottom layer of hardened, water-saturated plaster several mm thick, or a water-saturated synthetic sponge

occupying about 25% of the container volume. A hole (1 cm diameter) was drilled in the box lid and plugged with a rubber stopper. This hole allowed water to be added and/or substrate for the ants to process, if required. The nest entrance was a piece of PVC tubing (5-10 cm long and 1.5 cm internal diameter) tightly fitting a hole on one side of the box. Nest boxes were placed in trays having sides coated with Fluon™ (ICI Fluoropolymers, Exton, Pennsylvania) to prevent escape. This setting provided the ants with a nest, and foraging and refusal-dumping areas in each tray (Figure 3.1).

In order to obtain naïve workers for tests, groups of 20-30 workers of *A. cephalotes* and *Atta mexicana* placed separately in nest boxes without fungus were given mature (pigmented) pupae, and/or pharate workers and/or callow workers from queen-right, laboratory colonies with their native fungus (colonies greater than 2-years old, with tens of thousands of workers). More *A. cephalotes* workers were available for the experiments, so the observations of this chapter focused on this species mainly; observations on *A. mexicana* naïve workers were done only in chimeric nests combining both species (see below).

Pupae and callow workers were cleaned individually (under the dissectin microscope, with a pin) to eliminate adhering fungal fragments and then transferred to the nests described above with conspecific workers. In preliminary observations, mature workers ("nurses") accepted and protected conspecific pupae and callow workers from other colonies. Great care was taken not to leave any fungus particles on these individuals. However, I could not be absolutely sure that all fungus was stripped off. In this respect, preliminary observations showed that if there was any acceptable fungus present, nurse workers would rapidly consolidate and start cultivating it. Thus, nurse workers with pupae were routinely provided with plant material (see below), to detect residual fungus that would then be eliminated.

Under these conditions nurse workers engaged in protecting and nursing the pupae and callows: e.g. they piled them up, cleaned and licked them carefully, and later helped the callow workers emerge. In attine ants, adult emergence from the pupa requires assistance

from workers; unattended pharate adults are unable to emerge from the thin pupal envelope and die (Weber 1972). Through all the manipulations, the nursing nests were carefully and closely inspected so no maternal (native) symbiotic fungi were present at any time. The newly emerged workers were transferred to mini-nests within 36 h after their emergence. In the mini-nest, they were provided with fresh fragments (0.25 g.) of an actively growing fungus garden taken from different colonies to be tested. Larvae and eggs were eliminated by careful searches of each garden fragment. Under these conditions, "naïve" workers readily accepted the fungus provided and within days started collecting substrate and cultivating either the native or novel (switched) cultivars and building fungal gardens. The substrate provided was new growth of privet, *Ligustrium* sp., Bradford ornamental pear (*Pyrus calleryana*) leaves, and orange (*Citrus aurantifolia*) slices and fruit skin. Based on extensive preliminary observations, substrate was provided in amounts sufficient to sustain a constant increase in fungal garden biomass in mini-nests harboring native symbioses. Additional naïve workers were added to these incipient mini-nests as workers emerged from pupae over a one-month period. By identical procedures, I established mini-nests of *T. zeteki* workers cultivating an *A. cephalotes* cultivar. Due to limited worker availability not all mini-nests had the same number of workers: *Atta* mini-nests had an average of 25.6 (23-27) workers, whereas the *Trachymyrmex* mini-nests had an average of 14 (12-16) workers. The experimental ant-fungus combinations are listed on Table 3.1; four to six replicates were established for each ant-fungus combination. Fungus garden weight readings were taken after 74, 105, and 135 days. For the individual fungus gardens, nest boxes were open; ants were carefully removed from the fungus and put back on the colony's foraging tray; the fungus garden was placed on a weighing "boat" and rapidly weighed then placed back in the nest box.

Compatibility of workers of different *Atta* spp. and fungus garden growth. The behavioral flexibility of naïve workers enabled cultivar and nestmate switching. However, innate preferences constrained the experimental approaches of this study. When mixed together in mini-nests, naïve workers of *A. cephalotes* and *A. mexicana*

were highly tolerant of each other and coexisted peacefully over several weeks (Sánchez-Peña, unpublished observations). To have a more complete perspective of behavioral flexibility of naïve workers, I made additional parallel observations on the compatibility of non-conspecific naïve workers of *A. cephalotes* and *A. mexicana*, as determined by their cooperative agricultural behavior (if any) and fungal garden biomass changes over months of coexistence in the same nest. Thus, four mini-nests were established combining *A. cephalotes* and *A. mexicana* naïve workers [number of workers averaged 9.7 (range 9-11) for *A. mexicana*; and 15.5 workers (range 13-21) for *A. cephalotes*].

3.2.2 Numbers of Gongylidia Produced in Mini-nests of Ants Cultivating Native or Novel Cultivars.

Gongylidia counts. The number of gongylidia/mg of fungus garden was established by phase-contrast microscopic examination of fungal biomass from the experimental mini-nests and from queen-right colonies. For sample collection, gardens were considered to be composed of three horizontal strata or layers. The ants add new processed substrate (plant biomass in this case) to the top of the garden, which they then inoculate with tufts of fungal hyphae. Simultaneously they remove and discard from the bottom part of the garden exhausted biomass from which the gongylidia have been harvested. In this way, plant material inoculated with a fungus slowly moves from the top to the bottom of gardens, while the fungus matures (produces staphylae); the harvested staphylae therefore develop predominantly in the intermediate layer between the garden top and bottom. Thus, if the garden is considered to be made up of top, central, and bottom sections, of roughly equivalent size, then staphylae can be observed developing on the top and central two thirds of the fungal garden column. Biomass for gongylidia counts was collected from the central third of gardens after removing the top third of the fungus garden, which had a thickness that was variable, depending on the garden size. Five samples (about 10 cm^3) were taken from different randomly chosen points in the gardens, which were then thoroughly but gently mixed.

A sample of known weight (usually 1 g) of fungus garden was taken from this mix and blended in 30 ml of tap water in a Waring blender. Each blended sample therefore is the average of five samples. Fungi were blended during seven to ten "instant" bursts, applied by swiftly switching the blender on and off. Microscopic examination showed that this type of blending bursts dislodged the dense staphylae and separated the individual gongylidia, making it possible to count each. Staphylae are commonly formed by more than 60 densely clustered gongylidia; less blending did not allow separation of gongylidia from clusters, thus preventing accurate counting; more blending tended to disintegrate the individual cells. After blending, the gongylidia were counted using an improved Neubauer cell counting chamber and standard cell count methods (Caprette 2004). Either individual cells or small branches holding a few gongylidia were commonly observed. Both club-shaped and spherical cells were included in the gongylidia counts; the criterion was to consider as gongylidia only those cells clearly differentiated from the vegetative hyphae, especially if they were part of the branches forming the staphylae. The number of gongylidia/g of garden was determined for selected combinations of ant species and fungal symbionts (Table 3.2).

The number of gongylidia in gardens is the result of the interaction of their production rate and their consumption by the resident ants, mainly larvae and the queen. Besides the overall quality of substrate used by the ants, the number of larvae consuming the gongylidia is expected to be a major factor responsible for the number of such cells on fungal gardens. The larvae and the queen have long been assumed to be the main consumers of fungal biomass (gongylidia); workers are assumed not to depend on this food source for their energy needs (Cherrett et al. 1989). Thus, gardens with and without larvae were compared in this respect (Table 3.2) in order to estimate consumption of gongylidia by larvae in native , growing leaf-cutter colonies. The evaluations reported herein are the first to quantify and compare numbers of nutritive cells in gardens of fungus growing-ants.

Egg-laying by *Trachymyrmex* workers and gongylidia counts. *Trachymyrmex* workers cultivating an *Atta* fungus in mini-nests produced abundant eggs and larvae that they

raised to adult males. Raising these male larvae was expected to lower the numbers of gongylidia in the respective gardens. Workers of *Atta* and *Acromyrmex* do not reproduce in queenless nests; workers cannot develop their regressed ovaries. Fungus gardens of *Trachymyrmex* ants were only qualitatively observed for viability, stability, growth and gongylidia production; gongylidia were not quantified in these.

3.2.3 Data Analysis.

Gonglylidia counts. Because of the limited numbers of replicates/treatments from which gongylidia could be counted and compared, only the numbers of gongylidia of selected mini-nests and full colonies were compared. These results are shown graphically in Figures 3.3 and 3.4.

Fungus Garden Growth. I compared the slopes of the regression lines for weight garden changes among treatments. These slopes indicate fungal garden growth rate. Two analyses were performed: Pair-wise Analysis of Covariance (ANCOVA) using the software "JavaScripts E-Labs Learning Objects", University of Baltimore (Arsham 2004): (http://home.ubalt.edu/ntsbarsh/Business-stat/otherapplets/ANOCOV.htm). This analysis perfoms simultaneous regression analysis of selected pairs of treatments (i.e. ant/fungus combinationsin in mini-nests) and compares their regression slopes (growth rates). This test indicates if slopes are equal.

Additionally, I performed a non-linear least squares curve-fitting analysis, comparing simultaneously the slopes of the four treatment lines using the software (http://members.aol.com/johnp71/nonlin.html) from Interactive Statistics (http://www.statpages.net) by J. C. Pezzullo, Georgetown University (Pezzullo 2004). Average weight data were transformed to logarithms (Ln) for linearization. The regression model fitted the lines to go through the initial weight value (250 mg) at time zero. This analysis selects a treatment as reference model for slope and compares simultaneously the remaining treatments against the reference. Treatment 2 (*A. cephalotes*/*A. mexicana* workers cultivating an *A. cephalotes* fungus), which had the

graphically intermediate weight change line, was used as reference slope (Figure 3.2). All other slopes (treatments 1, 3 and 4) (Table 3.4) were compared to this reference slope.

The function to be fitted was:

y = 250 + time (days) * [p2 + (p1 if group=1) + (p3 if group=3) + (p4 if group=4)]

where p2 represents the slope of the line for treatment 2 (reference group), and p1, p3, and p4 represent the difference between the slope of treatment 2 and the slopes of treatment 1, treatment 3, and treatment 4, respectively.

In the JavaScript-compatible syntax, the function to be fitted was:

y = Ln(250) + x2*[((x1= =1)?p1:0) + p2 + ((x1= =3)?p3:0) + ((x1= =4)?p4:0)]

where x1 is the number assigned to each treatment for analysis: 1, 2, 3, or 4; x2 is the time, in days; and p2 is the reference slope, and p1, p3, and p4 are the difference between the slope of treatment 2 and the slopes of treatment 1, treatment 3, and treatment 4, respectively, as described above. In the JavaScript function, the expression (x1= =1)?p1:0 means "p1 if x1 equals 1, otherwise 0". This simultaneous curve-fitting analysis is relatively more rigorous than ANCOVA, since it avoids the "inflated alpha level" that always accompanies doing multiple pairwise significance tests (Sokal and Rohlf 1994), and also it bases the "error variance" estimate on all the data observations, increasing the precision of the estimate, and hence the degrees of freedom in the analyses (making it more powerful at detecting effects) (J. C. Pezullo, personal communication).

3.3 RESULTS

3.3.1 Acceptance.

The naïve *Atta* workers readily accepted the non-native *Trachymyrmex* symbiotic fungus. Naïve *Atta* workers exposed to native *Atta* fungal symbionts also accepted these *Atta* fungi as normally occurs. The naïve *Trachymyrmex* workers also readily accepted the *A.*

cephalotes symbiont. Acceptance was indicated by a) the ants' persistent arrest on or by the fungus; b) the structured piling-up, grooming and licking of the fungus, and especially c) the stereotypical cultivation behaviors (substrate cutting, processing, manuring and incorporation into the fungus garden). Weber (1972) provides a detailed description of substrate processing in attine ants.

In contrast, rejection by attine workers of cultivars was indicated as: a) the ants'meticulous chewing of such rejected cultivars; b) active relocation of fungal pieces to nests corners and placement on the "dump", along with carcasses and other refuse material; c) abandonment of such fungi.

3.3.2 Stability.

The ants cultivated the fungus provided in all mini-nests in all types of experimental associations. These associations were stable for at least four months and many of the mini-nests in all four treatments thrived for more than 10 months until the workers gradually died off.

3.3.3 Fungal Weight Changes.

Figure 3.1 shows the trends in fungal garden weight changes in the mini-nests for the different symbiont combinations. Non-linear least squares curve-fitting analysis indicated significant differences between the slopes of fungal weight change over time (Table 3.5). The slope of the weight increase (rate of growth) for the *Trachymyrmex* fungal symbiont cultivated by *A. cephalotes* workers was significantly less than the slope in all three *Atta* ants/*Atta* fungus combination ($p<0.05$). The fungus growth rate in mini-nests combining *A. cephalotes* and *A. mexicana* workers with an *A. cephalotes* fungus was significantly less than those composed of *A. cephalotes* workers only/*A. cephalotes* fungus ($p=0.028$) or *A. cephalotes* workers only/*A. mexicana* fungus ($p=0.039$). There were no significant differences between the fungus growth rates (slopes) for *A. cephalotes* workers cultivating either an *Atta cephalotes* or an *A. mexicana* fungus. The analysis of covariance (ANCOVA) also indicated a slower fungus growth rate for the *Trachymyrmex*

fungus/*Atta* ant combination, but did not detect significant differences among the three treatments of *Atta* spp. workers cultivating *Atta* fungal symbionts (Table 3.4).

In summary, all ant/fungus associations were similarly stable in their duration. However, despite the stability of the associations, the fungal growth rate for the combination of *A. cephalotes* workers cultivating a *Trachymyrmex* symbiotic fungus was significantly less than those in the associations of *Atta* workers and *Atta* cultivars (Fig. 3.1; Table 3.5). The weight trends for the fungal symbionts of *A. mexicana* and *A. cephalotes* were virtually identical and showed a steady increase (almost 800%) over four mounths. In the combination of *A. mexicana* and *A. cephalotes* workers, the *A. cephalotes* fungus grew less but still increased almost 700% in weight, indicating a high level of social compatibility between workers of these two species and of workers and symbiont. In contrast, the fungus in the novel *Atta* ants/*Trachymyrmex* fungus combination increased only 200% in weight over this time period (Fig. 3.1).

3.3.4 Gongylidia Counts.

Gongylidia counts are time-consuming. This limited the number of mini-nests and colonies that were analyzed. Thus it was only possible to compare the average number of gongylidia in a limited number of combinations of ants with novel and native symbionts. Among the mini-nests with the novel ant-fungus combinations, gongylidia counts were highly variable, and on average much smaller than in the native, *Atta* ants/*Atta* fungus symbioses [185.8 (std. error 143.89) vs. 673 (std. error 13) gongylidia/mg, respectively] (Figure 3.2). Another notable fact was that two of the six gardens in the novel *Atta* ant/*Trachymyrmex* cultivar mini-nests showed no gongylidia at all; in these cases, although the fungal gardens did increase in weight, they were practically “sterile”, in that they produced no nutritious cells.

In the native *Atta-Atta* fungus association, when workers but no larvae were present, the gongylidia accumulated on gardens in very high numbers as shown in this work (Figure 3.3); for example, the fungus garden of the queenless colony became an authentic mass of

such cells (1966 gongylidia /mgr) due to the lack of consumption of the same since workers, but no larvae, were present. Thus, a general conclusion is that the consumption of gongylidia by workers is very small. The gongylidia counts in the five normal, native *A. cephalotes* colonies (growing colonies with queen, larvae and thousands of workers present) yielded rather consistent values across colonies, 65-133 cells/mgr. This apparent effect of larvae (or, less likely, the queen) on gongylidia number was very striking: the native mini-nests (*Atta* ants/*Atta* fungus) and the colony without queen or brood, had gongylidia numbers approximately 10 to 30 times higher than those of the native, normal functional colonies where abundant brood was present (Figure 3.2). A preliminary consumption rate of 80-90% of the gongylidia production can thus be estimated for native, normal laboratory colonies of *Atta* in the growing (ergonomic) phase (having more than a few hundred workers: see Chapter 4).

3.4 DISCUSSION

Plant substrate in mininests. In this work ants were maintained on a diet of young leaves and shoots of privet (*Ligustrium*), and orange slices and peel. This diet was perhaps biased towards leaf-cutter preferences, and this could in theory have affected the development of the *Trachymyrmex* fungus. Food substrates were chosen due to their availability. *Trachymyrmex* workers are markedly inclined to use insect feces and fallen inflorescences (catkins) (Weber 1972; Holldobler and Wilson 1990) and neither was regularly available; therefore they were not used. Besides, when available, *Atta* workers never picked up nor used these substrates. On the other hand, both ant genera were provided with orange slices; *Trachymyrmex* workers are very attracted to orange pulp, while *Atta* seemed to be very attracted to the white inner peel of oranges. These preferences were not evaluated. Oat flakes and oatmeal (commonly used to maintain lower attine colonies in the laboratory, i.e. Adams et al. 2000) were sprinkled on colonies and they were available to all ants, but they had a very poor to nil attractiveness to *Atta* workers. Thus they were not relied upon for fungus maintenance and foliage and oranges were provided as mentioned. If no other substrate is available, *Atta* workers will use oat flakes, but these will produce a withish, fluffy appearance on *Atta* fungus gardens. This

aspect is completely different from gardens in nature or in the laboratory when plant tissue is used. For these reasons I considered that using oat flakes and oatmeal could seriously affect development and growth of leaf-cutter gardens, especially over long periods of time. Also, plant sap and juices are essential to sustain the energetic requirements of *Atta* workers (Cherret et al.1989).

This diet of fruit and leaves allowed the maintenance of two thriving *Trachymyrmex zeteki* colonies over more than two years. These colonies were always in the population range of field colonies (≈ 300 workers) (Weber 1972). Stradling and Powell (1986) also maintained *Trachymyrmex zeteki* colonies over more than one year in the laboratory using *Ligustrium* leaves. I considered unlikely that this diet could have affected negatively the development of the *Trachymyrex* fungus.

Workers acceptance of symbiotic fungi. Observations on the acceptance of fungal cultivars by *Atta* and *Trachymyrmex* showed that "naïve" workers raised on their respective native fungi accepted novel symbionts. These findings indicate that the fungus used as food for worker larvae has little, if any, posterior effect upon short- and long-term acceptance of novel cultivars as symbionts by naïve adult workers, along with their cultivation by means of the innate, specialized behaviors. Thus, workers are not instinctively programmed to accept and tend only the particular fungus they were raised on, or the fungus that their lineage cultivates in nature. Instead, there appears to be a critical (sensitive) early period, somewhat similar to imprinting (Lorenz 1981; Dejean 1990) encompassing a few days after emergence from the pupa, during which workers assimilate and internalize signals from the cultivar they are exposed to, establishing this as their own fungal symbiont. This is a normal process ocurring continuosly in colonies as new workers eclose.

A customary definition of imprinting characterizes it as a learned response developed over a genetically determined, limited, usually early period in life, resulting in an irreversible preference even over the natural or original source of stimulus, i.e., a duckling imprinted to follow a balloon as its mother will follow it even if provided with

the choice to follow either its actual mother or the balloon (Lorenz 1981). In this work, I detected that there is an early sensitive period when symbiotic fungus cues are identified and learned, since workers experienced with the native cultivar will never accept (cultivate) a different (*Trachymyrmex*) symbiont. A full comparison of imprinting and traits like the early sensitive period in ants and vertebrates would not be simple since *Atta* workers deprived of a symbiotic fungus will only live from five to 25 days (Silva et al. 2003; Sánchez-Peña, unpublished observations) as opposed to a lifespan of several months when they cultivate *either* the native or a novel fungus, as shown here. Thus, it is difficult to determine the duration of the early sensitive period of ants to the fungus, and consequently the classical parameters of imprinting are difficult to apply conclusively to this system.

After this imprinting-like event, workers develop outwardly native agricultural behaviors towards either cultivar, native or switched. Nonetheless, despite the seemingly native and permanent acceptance (i.e., cultivation) of novel cultivars by ants shown here, the switched symbiosis suffered significant, deleterious consequences regarding fungus garden development. The symbiotic fungi of *Trachymyrmex* are conspicuous producers of gongylidia in their natural associations (gardens) with ants and also in artificial culture on agar plaques when workers are absent (Weber 1972; Sánchez-Peña, unpublished observations), but the same *Trachymyrmex* cultivar produced gongylidia poorly when propagated by *Atta*. The reduced number or complete lack of gongylidia in some of these mini-nests (average=186, vs. 673 in *Atta* ants-*Atta* fungus mini-nests) and the reduced fungal weight increase over time in the novel symbioses, suggest important mismatches, of yet unknown nature, between the switched symbionts. At this point it is possible to predict a severe effect on *Atta* spp. colonies, should they collect and cultivate a *Trachymyrmex* symbiotic fungus in the field. These mismatches probably generate intense selection pressure and evolution of imprinting-like mechanisms to prevent use of different cultivars. This results in the strong rejection behaviors of *Trachymyrmex* cultivars by experienced *Atta* spp. workers. The rejection behavior for a particular cultivar is influenceable by learning, not innate. The novel cultivar rejection by workers

experienced on the native cultivars occurs even if this results in their death when they do not have access to any other cultivar (Sánchez-Peña, unpublished observations).

Negative effects of fungal switch. The underlying nature of the described mismatches between *Atta* ants and *Trachymyrmex* fungus are unknown. These might be biochemical incompatibilities (Bot et al. 2001) residing in the *Atta* fecal materials used to manure the fungus, in their saliva, or in their digestive enzymes. It is also possible that the cultivating behavior of *Atta* workers could be detrimental to the *Trachymyrmex* fungus. Alternatively, it cannot be ruled out that it is the fungus that initially elicits the feedback mismatch on the novel symbiotic ants. Perhaps the mismatches are multiple and simultaneous for both novel symbionts, ant and fungus.

The comparisons between cultivars of different *Atta* species also indicate that these symbiotic fungi are functionally very similar or perhaps identical in parameters such as growth rates, nutritional value, and biomass transformation and efficiency. This supports the concept that extremely similar and largely interchangeable fungal cultivars, identifiable by genetic markers, exist among the leaf-cutting ants (Chapela et al 1994; S. Rehner, personal communication); thus, in Brazil, several non-sympatric species of *Atta* and *Acromyrmex* cultivate essentially the same fungus (Silva-Pinhati et al. 2004) indicating possible regular horizontal transmission of cultivars, or, alternatively, highly stable and conserved cultivars after speciation. There is also evidence of some level of horizontal transmission between *Ac. echinatior* and *Ac. octospinosus* in Panama (Bot et al. 2002). The apparent horizontal transmission of these highly-derived fungal symbionts among leaf-cutter species reinforces the idea of versatile cultivars within this clade, which must be able to fulfill the large nutritional demands of the populous colonies of *Atta* and *Acromyrmex*.

Future research on attine-cultivar coadaptations should expand on the parameters described here (gongylidia amounts and fungal biomass change on different attine symbioses, natural and experimental), as well as on additional characteristics of the ant-fungus interface; for example effect of cultivars on substrate foraging and processing,

symbionts' longevity, metabolic rates, enzymatic activities, and genetic stability of cultivars over time. The imprinting-like behavior and the acceptance of naïve ants during their initial exposure to symbiotic fungi need to be investigated more thoroughly.

Figure 3.1. Ant colony culture system. From left, three *Atta mexicana* colonies cultivating a *Trachymyrmex* symbiont, and one native *A. mexicana* colony. Note differential symbiotic fungus pigmentation in box at extreme right.

Figure 3.2. Changes in average fungal weight of mini-nests containing different workers/fungal symbiont combinations. Four replicates (mini-nests)/treatment were averaged, except for the *Atta cephalotes* workers cultivating a *Trachymyrmex zeteki* fungal symbiont treatment which involved six replicates. Line slopes with a different uppercase letter are different (non-linear least squares curve-fitting analysis: see Table 3.5). Line slopes with a different lowercase letter are different (ANCOVA), $P < 0.05$.

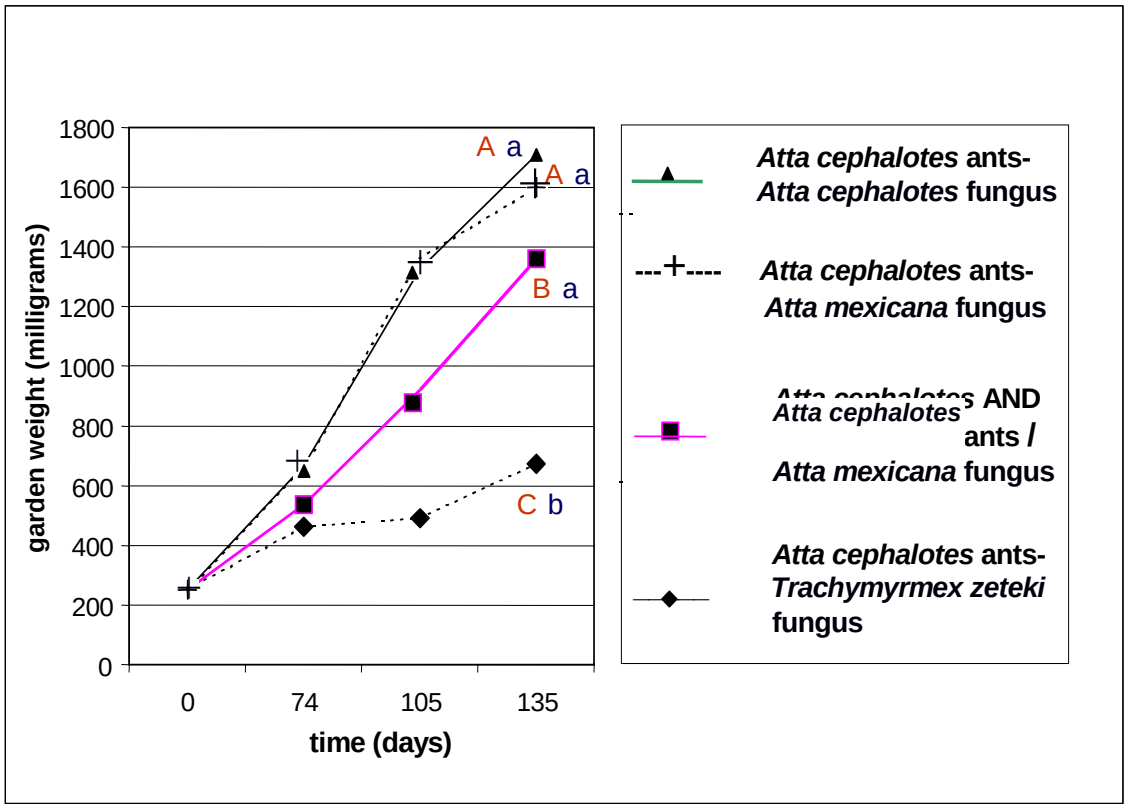


Figure 3.3. Numbers of gongylidia/milligram in fungal gardens from different combinations of fungal and ant symbionts in mini-nests.
M1-M4 = *Atta cephalotes* workers cultivating a *Trachymyrmex zeteki* fungal symbiont; **M5** = *A. cephalotes* workers with an *Atta mexicana* fungus; **M6** = *A. cephalotes* workers with an *A. cephalotes* fungus.

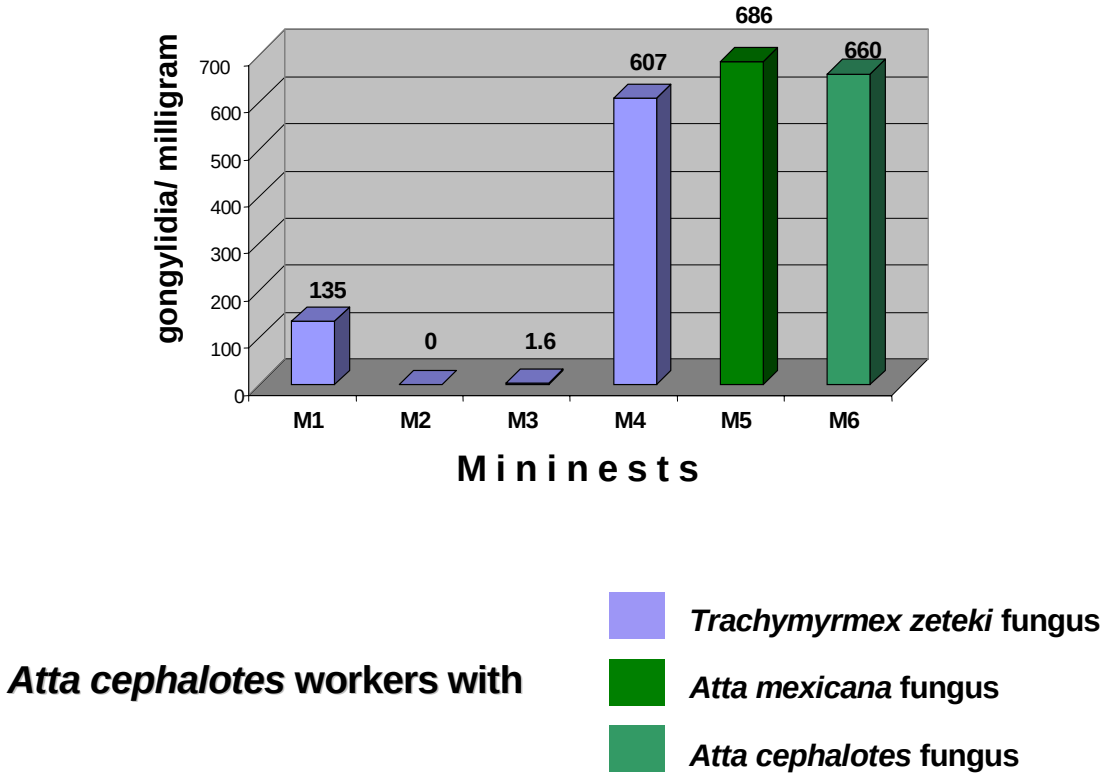


Figure 3.4. Numbers of gongylidia/milligram in fungal gardens from different combinations of fungal and ant symbionts in colonies.
C1 = *Atta mexicana* queenright colony with an *Acromyrmex octospinosus* cultivar; **C2** = *A. cephalotes* queenright colony with an *A. mexicana* cultivar; **C3-C7** = *A. cephalotes* native colonies; **C8** = *A. mexicana* colony and fungus, colony queenless for 7 months (queen died thus no more brood produced).

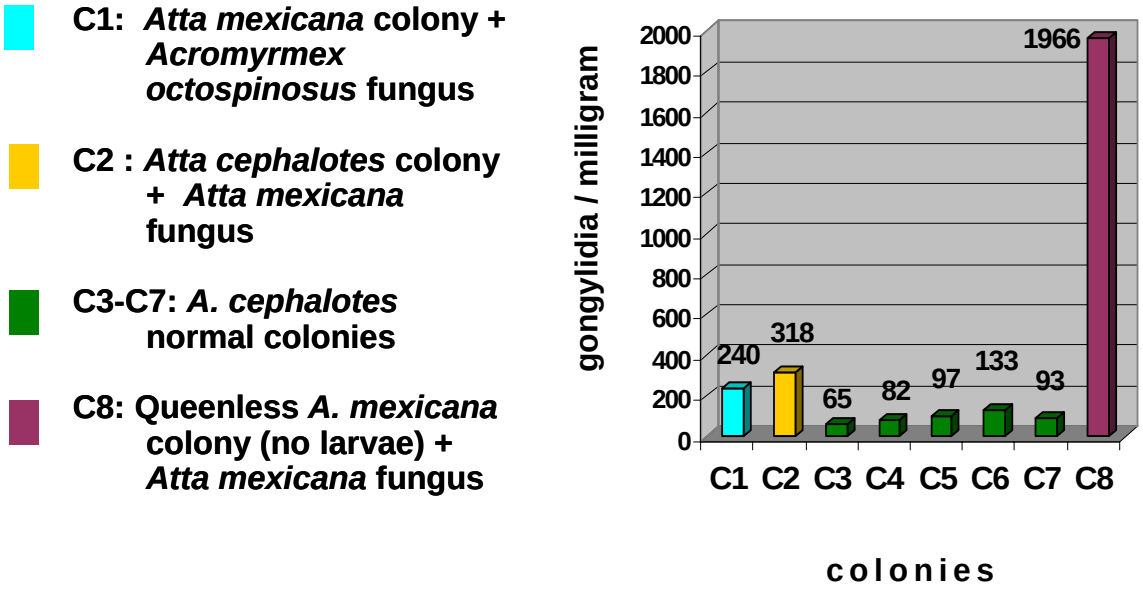


Table 3.1. Ant-fungal combinations in native and symbiont-switched mini-nests analyzed to determine changes in fungus garden weight.

Ant species	Fungal symbiont	Number of replicates
Atta cephalotes	*Atta cephalotes*	4
Atta cephalotes	*Atta mexicana*	4
Atta cephalotes	*Trachymyrmex zeteki*	6
Atta cephalotes + *Atta mexicana*	*Atta cephalotes*	4

Table 3.2. Ant-fungal combinations, in native and symbiont-switched queen-right colonies or queen-less mini-nests, analyzed for numbers of gongylidia on gardens.

Ant species	Fungal symbiont	Number of replicates
Atta cephalotes	*Atta cephalotes*	5 colonies
Atta cephalotes	*Atta mexicana*	1 colony
Atta mexicana	*Acromyrmex octospinosus*	1 colony
Atta mexicana (colony queenless for 9 months)	*Atta mexicana*	1 colony
Atta cephalotes	*Trachymyrmex zeteki*	4 mini-nests
Atta cephalotes	*Atta cephalotes*	1 mini-nest
Atta cephalotes	*Atta mexicana*	1 mini-nest

Table 3.3. Weight change for fungal gardens: mini-nests with ant/fungal combinations.

Treatments and replicates	Date of garden weighting and weight (gr)			
	day 0	day 74	day 105	day 135
CT: *A. cephalotes* ants/ *Trachymyrmex* fungus				
CT 1	0.25	0.26	0	0
CT 2	0.25	0.61	0.98	1.27
CT 3	0.25	0.94	1.49	2.42
CT 4	0.25	0.69	0.23	0.065
CT 5	0.25	0.1	0	0
CT 6	0.25	0.18	0.24	0.28
Average and (SE)	0.25 (0)	463.3 (135.8)	490.0 (248.3)	672.5 (401.5)
BC: *A. cephalotes* and *A. mexicana* ants/*A. cephalotes* fungus				
BC 1	0.25	0.67	1.54	2.25
BC 2	0.25	0.18	0	0
BC 3	0.25	0.18	0	0
BC 4	0.25	1.12	2.14	3.19
Average and (SE)	0.25 (0)	537.5 (225.9)	920.0 (545.0)	1360 (808.3)
CC: *A. cephalotes* ants/ *A. cephalotes* fungus				
CC 1	0.25	0.84	1.71	2.37
CC 2	0.25	0.56	1.55	2.66
CC 3	0.25	0.2	0	0
CC 4	0.25	1.07	2.05	2.09
Average and (SE)	0.25 (0)	667.5 (187.5)	1327.5 (454.6)	1780 (604.6)
CM: *A. cephalotes* ants/ *A.mexicana* fungus				
CM 1	0.25	0.35	0.4	0.72
CM 2	0.25	0.2	0.59	0.23
CM 3	0.25	1.06	2.08	2.75
CM 4	0.25	1.03	2.38	2.77
Average and (SE)	0.25 (0)	660 (224.4)	1362 (506.06)	1617.5 (667.17)

Table 3.4. Average garden weights for ant/fungus combinations in mini-nests, and parameter estimates (slope and intercepts) for regression equations of treatments (Analysis of Covariance).

	Weight (Days since start of experiment)				Slope (SE)	Intercept (SE)
Ant-fungus combination	**0**	**74**	**105**	**135**		
***A. cephalotes* ants/*Trachy* fungus**	250	463	490	672	2.90 (0.48)**a**	240.93 (43.5)
***A.cephalotes*-*A. mexicana* ants/ *A. cephalotes* fungus**	250	537	920	1360	7.85 (1.90)**b**	149.94 (177.5)
***A. cephalotes* ants/ *A. cephalotes* fungus (control)**	250	667	1327	1708	10.82 (2.23)**b**	138.56 (208.7)
***A. cephalotes* ants/ *A. mexicana* fungus**	250	660	1362	1601	10.36 (2.15)**b**	158.44 (201.1)

Slope (weight increase rate) values followed by the same lowercase letter are not significantly different (p=0.05). *Trachy = Trachymyrmex zeteki.*

Table 3.5: Summary of non-linear least squares curve fitting of fungus garden growth data.

Ant-fungus combination	slope	+/- std. error of slope	p value
T1. *A. cephalotes* ants/ *Trachy* fungus	0.007192	0.000917**a**	0.0006
T2. *A. cephalotes* & *A. mexicana* ants/ *Trachy* fungus	0.012153	0.00064**b**	0.0000*****
T3. *A. cephalotes* ants/ *A. cephalotes* fungus (control)	0.014608	0.000917**c**	0.0280
T4. *A. cephalotes* ants/ *A. mexicana* fungus	0.014413	0.000917**c**	0.0390

***** Reference group

Average weight data were Ln-transformed for linearization. *Trachy=Trachymyrmex.* The treatment with a curve intermediate in position [treatment 2 (T2), combining *A. cephalotes* & *A. mexicana* ants with an *A. cephalotes* fungus] (see Fig. 3.1) was used as reference group. Significance value for reference group indicates whether its growth rate is different from zero; p values for the other treatments indicate significance respective of the slope of the reference group. Growth rates (slope values) followed by the same lowercase letter are not significantly different.

Chapter 4: Experimental Establishment of Functional Colonies of *Atta mexicana* Cultivating Native or Novel (*Trachymyrmex*) Fungal Symbionts: Fitness Effects and Worker Preference for Cultivars

Synopsis: In this chapter I sought to detect possible constraints at the colony level preventing intergeneric, macroevolutionary fungal cultivar exchange among higher attine ants (*Trachymyrmex* and *Atta* spp.). I established functional, queenright colonies of *Atta mexicana* ants cultivating either their own fungal symbiont (native), or switched to a *Trachymyrmex zeteki* fungal symbiont (novel). I compared these native and switched *A. mexicana* colonies for worker numbers and worker size. I determined also the cultivar preferences in workers from both native and switched, mature colonies. The switch of symbiotic fungus caused specific, severe adverse effects on worker number and size. A learned behavioral component was found controlling worker acceptance of fungal cultivars; this learned component allowed the long-term cultivation of extraneous symbionts by *A. mexicana* colonies.

4.1 INTRODUCTION

Cultivar evolution is believed to have played a major role in inducing ecological and macroevolutionary innovations in attine ants (Wetterer 1994; Mueller 2002). Wilson (1986) asserted that this key adaptation in leaf-cutter ants, which allowed "efficient utilization of almost all forms of fresh vegetation," was so unusual and successful "that it can properly be called one of the major breakthroughs in animal evolution." Exquisitely adapted and productive strains of fungal symbionts must have played a major role in such a breakthrough. It has been estimated that the subterranean fungus gardens of colonies of leaf-cutters in the genus *Atta* can weigh almost 150 kg (Stahel 1943), and individual *Atta* colonies can produce almost 5 kg of ants (Weber 1972).

The higher attines constitute a natural group, which as originally defined by morphology (Weber 1972) includes two main clades: the rather saprophagous, non leaf-cutter *Trachymyrmex-Sericomyrmex* clade and the leaf-cutter clade (*Atta* and *Acromyrmex*) (Currie et al 2003). In nature, the fungi cultivated by the higher attines are restricted to two clades, which correspond to the two ant clades (i.e. the *Trachymyrmex* symbiosis cultivars and the leaf-cutter symbiosis cultivars). Strains are genetically very similar within these fungal clades, particularly those in the leaf-cutter symbiosis. Extensive sampling indicates that symbiont fidelity is almost total in the higher attines; each higher attine clade cultivates only fungi from their respective strain clusters (Chapela et al. 1994; Bot et al 2001; Currie et al 2003; U. Mueller, personal communication; S. Rehner, personal communication).

There are many opportunities for exchanges (switches) of fungal symbionts between the *Trachymyrmex* symbiosis and the leaf-cutter symbiosis in nature, considering the biology, abundance, sympatry and proximity of nests of these two higher attine groups. For example, *Trachymyrmex* colonies commonly nest and forage on *Atta* mounds (Weber 1972; Sánchez Peña, personal observations). In addition, expanding *Atta* colonies can cover more than 100 m^2 and are likely to overrun colonies of other attines (personal observations). However, despite extensive sampling, no symbiont switches have ever been reported among natural colonies in the field (Chapela et al. 1994; Bot et al., 2001; Mueller 2002; Silva-Pinhati et al. 2004; U. Mueller, personal communication; S. Rehner, personal communication). This symbiont fidelity or conservatism in the higher attines indicates probable instantaneous, severe ecophysiological constraints against breaking this symbiont alliance through switches. Thus, a parsimonious hypothesis is that functional coadaptations between fungi and the highly derived leaf-cutting ants are responsible for the preclusion of symbiont change in nature. However, the natures of these theoretical adverse effects, and the coadaptations responsible for cultivar fidelity in the higher attines are completely unknown.

There are very few empirical data on cultivar switches to support theories regarding possible ant-cultivar coadaptations in the higher attines. An anecdotal report (Powell and

Stradling 1986) mentions that one decaying *Trachymyrmex zeteki* Weber colony had a poorly-growing fungus garden; when this was replaced with a piece of an *Atta cephalotes* (L.) fungus garden, the *Trachymyrmex* ants cultivated the new fungus and regained what apparently was native colony growth. However, this observation involves only one colony and no quantitative data are provided.

Bot et al. (2002) described the variability of acceptance and rejection of cultivars, for intra- and interspecific switches among workers of the sister species *Acromyrmex octospinosus* Reich and *Acromyrmex echinatior* Forel. In this particular short-term study, both ants and fungal strains were phylogenetically very closely related, and the interpretations of cultivar discrimination patterns and, particularly the selective pressures leading to such patterns, appeared more complex. There is evidence that under natural conditions, the lower attines occasionally import or aquire novel cultivars (even from free-living strains). However, there is no information on constraints or effects of these acquisitions (Mueller et al. 1998; Green et al. 2002; Mueller 2002). In a study of cultivar transfer in inter- and intraspecific pairings of colonies of the sister species *Cyphomyrmex muelleri* Schultz and Solomon and *Cyphomyrmex longiscapus* Weber (when *C. muelleri* was deprived of fungus garden), only 25% of interspecific pairings resulted in cultivar transfer, versus 100% and 78% in intraspecific pairings (Adams et al. 2000). These authors report also that the respective fungi of these ants are distantly related and that *C. longiscapus* will not readily accept gardens of *C. muelleri*. Also with *C. muelleri*, Mueller et al. (2004) showed acceptance and choice of novel cultivars along a phylogenetic cline; novel cultivar acceptance was directly related to phylogenetic relatedness of the original, lost cultivar. Intraspecific, between-nest transfer of fungal cultivars by means of colony raids has been observed for the leaf-cutting ants *Atta sexdens rubropilosa* Forel (Autuori 1950) and *Acromyrmex versicolor* Pergande (Rissing et al. 1989). However, Adams et al. (2000) contend that "no study has documented raiding *between* attine species or genera under natural conditions".

In order to detect possible constraints on fungal symbiont exchange in the higher attines, I established queenright *Atta mexicana* colonies cultivating either a native fungal

symbiont or a switched (novel) fungal symbiont of *T. zeteki*, to evaluate cultivar effects on workers' growth in sizes and numbers as well as the cultivar preference of these workers towards native and novel symbiotic fungi.

For these comparisons, it is important to understand how *Atta* colonies grow. The growth of ant colonies has been separated into three phases or stages with respect to worker number and size, and production of sexuals (winged, fertile males and females) (Wilson 1983a, b; Hölldobler and Wilson 1990; Wetterer 1999). First, at the founding stage, the newly mated queen seeks a suitable nest site and produces eggs. In *Atta*, the new queen also expels the pellet of fungal inoculum carried from the maternal nest's fungus garden. She starts cultivating this fungus with her fecal materials. She concurrently rears the first adult worker generation (a few dozen workers). These first-cohort workers are usually of smaller size than those found in older colonies (Wilson 1983b). Next, at the ergonomic stage, workers of widely different sizes appear in *Atta*, and they take over the tasks of foraging, nest enlargement, brood care, and feeding the queen. The term ergonomic is used because at this stage ant colonies start a process of optimization of colony tasks (Wilson 1980, 1983a, 1983b). The ergonomic stage in *Atta* colonies begins at about four months of age (in the field and the laboratory), as new castes of larger and smaller workers are rapidly added, and division of labor develops among differently sized workers. These colonies also grow exponentially in worker number. Soldiers (the largest worker size, usually specialized in defense; head width $\geq$ 3.5 mm) appear after about six months in several species of *Atta* (Weber 1972; Wilson 1983b). Finally, at the reproductive stage, when the colony has reached a certain population size, winged virgin queens and males are produced, which will form the next generation. In this work, the ergonomic phase is considered to include the lapse between the founding and reproductive stages, although the full range of worker sizes appears, as mentioned, at about six months of colony age.

It is important to mention that regarding worker size distribution, there is no difference between the full ergonomic and reproductive stages of colonies (Wilson 1983a, 1983b;

Hölldobler and Wilson 1990; Wetterer 1999). Workers of *Atta* species are extremely polymorphic; their head width (HW) ranges from 0.6 mm to more than 5 mm (Weber 1972; Hölldobler and Wilson 1990);

4.2 MATERIALS AND METHODS

4.2.1 Establishment of Colonies.

About 250 queens of *A. mexicana* were collected in Monterrey, in the Mexican state of Nuevo León, on 4 July 2001, after a major nuptial flight. They were collected while they were digging their incipient nests. Queens were placed in artificial nests previously described (Chapter 3). They were closely observed to detect expulsion and cultivation of the fungal inoculum carried in their infrabuccal pellet. In the colonies to be switched, these early fungal gardens were removed 3-8 days after their inception, and the queens were given an actively growing fungus fragment from the top of fungus gardens of either a *Trachymyrmex zeteki* or an *Acromyrmex octospinosus* colony. In this way I was able to establish two functional, symbiont-switched colonies of *A. mexicana*, one cultivating the *Ac. octospinosus* symbiont and the other the *T. zeteki* symbiont. By analogous procedures, after the mating flight of the next year (2002), I established six more *A. mexicana* colonies: three native, and three switched to a *T. zeteki* fungal symbiont. Thus, a total of four *A. mexicana* colonies having the *T. zeteki* fungal symbiont, one cultivating the *Ac. octospinosus* symbiont and four native *A. mexicana* colonies, were available for study. Because fungi of *Acromyrmex* and *Atta* (leaf-cutters) are extremely close phylogenetically in Brazil (Silva-Pinhati 2004) and in Panama (S. Rehner, personal communication), I expected that the switch to a *Trachymyrmex* fungus would have more informative consequences. Therefore my observations focused mostly on *Atta* ants cultivating the *Trachymyrmex* symbiont. Observations were conducted on these particular colonies for over 28 months (one native and one switched colony from 2001), and for over 16 months for one native and three switched colonies from 2002.

4.2.2 Parameters Evaluated in Native and Fungal Symbiont-switched Colonies of *A. mexicana.*

The native and switched colonies were evaluated for the following: 1) appearance of the fungal garden; 2) time required to grow a known volume of fungus garden; 3) changes in queen and fungus garden weights in six colonies after more than a year; 4) total worker number; 5) worker size (head width) distribution; 6) cultivar preference of workers raised from larvae on either fungal symbiont.

Appearance of the fungal garden. The fungus appearance in the six 2002 colonies was recorded, as were the overall texture, density, appearance of gongylidia, and presence of fecal droplets in the nest boxes. Fecal droplets appeared as brown spots on all surfaces where the ants walked on; these are territorial markers (Hölldobler and Wilson 1990); they could have other functions too.

Change in queen and fungus garden weights. 128 and 228 days after their establishment in the laboratory, the queens and fungus gardens of the six 2002 experimental colonies were weighed. For the individual fungus gardens, nest boxes were open; ants were carefully removed from the fungus and put back on the colony's foraging tray; the fungus garden was placed on a weighing boat, rapidly weighed, then placed back in the nest box. Queen and fungus garden weights were compared by means of the Mann-Whitney test (Sokal and Rohlf 1994; Lejarza and Lejarza 2004).

Time required for the ants to fill the nest box provided with a fungus garden. I recorded, for the six 2002 native and switched colonies, the time required to build a fungus garden that filled the nest box provided (423.5 cm^3) (Fig. 4.3). Colonies were started from founding queens as described. Time periods (in days) were compared by means of the Mann-Whitney test (Sokal and Rohlf 1994; Lejarza and Lejarza 2004).

Trends in total worker number. Several complementary approaches were used to estimate total worker numbers in colonies, especially in native colonies that very populous having thousands of workers. Fungus-switched colonies were much less

populous (see below). The worker size distributions of two *Trachymyrmex* spp. are also reported here, as this genus was the donor of the novel fungal symbiont for switched colonies.

a) Most workers of the smaller size classes do little, if any, work outside the colony, and they stay and work largely in the fungus garden (Weber 1972; Sánchez Peña, personal observations). Therefore, fungal gardens of both native colonies were sampled and I estimated the number of workers per volume of fungus. Samples of fungus gardens (3 x 3 x 2.5= 22.5 cm^3) were collected from the nest boxes from one 16-month old and one 28-month old native colony and the workers inside these garden samples were counted. The fungal samples taken from these colonies represented 5% of the total fungus garden. It was anticipated that garden volume and worker number would be correlated, since fungal cells are the only food of attine larvae (Weber 1972). All workers were manually extracted from these fungus gardens with forceps under a dissecting microscope. In this way I obtained an estimate of the number of workers present per volume of fungus (in parts of the fungus gardens not harboring brood piles; see below).

b) Personal observations in laboratory colonies of *Atta* spp. indicated that many of the smallest workers (< 1 mm) are usually clumped near the brood that is accumulated frequently (but not always) near the surface of the nest box. These smallest of workers on the brood piles were counted through the clear plastic.

c) Workers of the largest size classes (largest majors and soldiers) were visually searched for, and all were counted, in the entire colonies.Nest boxes were opened to collect these largest workers that usually stayed inside the nests. This directed search was necessary since, in *Atta* colonies, workers measuring more than 3.2 mm HW represent only 6% of the worker numbers, but they make up 41% of the total workers biomass (Wetterer 1999).

d) The total number of workers in the switched colonies was counted; the colonies had 200-400 workers only.

Head width (HW). The ants' maximum HW in mm was measured with a Mitutoyo™ caliper (Mitutoyo Corp., Tokyo, Japan) under the dissecting microscope. For *A. mexicana* I took this measurement at the widest point of the head (eye level or above, or occipital lobes) by slowly closing the measuring clamps on to the ant's head until it was held barely but rather firmly. Variations of the grip on the ant's head with the caliper were very small (<0.05 mm). Due to the presence of lateral hairs in the ant's head, care was taken not to stop the caliper's measuring brackets at the hair's tip but at their base. For *T. zeteki*, the measurements were made at the tip of the solid, hard spines projecting from the sides of the head.

Native colony from 2002. I obtained the size distribution for 178 workers collected alive in random samples of the fungus garden, as described, as well as from additional randomly collected workers from the largest native colony (28 months old). Dead workers were also randomly collected from the colony dump. They were collected from the surface of dumps to obtain recently dead ants (a few weeks old at most).

In this way, more than 200 workers were secured and measured from this colony, and from the *T. zeteki* colony. I generated graphs of the size distribution for these colonies (Fig. 4.1). *Trachymyrmex* is considered to be an essentially monomorphic or slightly polymorphic genus (Weber 1972, Hölldobler and Wilson 1990; Beshers and Traniello 1994).

Switched colony from 2002. Some reports of worker size in *Atta* spp. have measured a few hundred or more workers per colony, which is well above the number of workers in each of the four switched colonies in the present work. Measuring that many workers would have destroyed these valuable switched colonies. Thus, for the 2002 switched *Atta* and the native *T. zeteki* colonies, I measured the carcasses randomly recovered from the surface of dumps to avoid destroying the colonies by worker elimination. Similar mortality rates are assumed among workers of different sizes.

Additionally, after having measured with the caliper more than 800 workers from the various colonies, of which more than 700 were of the smaller size category (0.6-1.5 mm HW), a visual image was obtained (for a few days at least) that allowed the workers to be classified by sight in a HW category usually within a 0.1 mm range. Then, the HW worker sizes were estimated visually in the switched colonies; it was possible to obtain an overall estimate due to the small worker number. Subsequently I calculated the approximate ratio of worker HW categories for all *A mexicana* colonies. This estimation was done to avoid destructive sampling (measurements of killed workers) of these fragile colonies.

4.2.3 Worker Preference for Native and Novel Cultivars.

The experimental set up can be divided according to the several steps required for its establishment.

Starvation of test workers: workers from 2001 colonies (native and switched ones) were isolated for 48 hours without fungal symbionts of any kind.

Two types of fungi, from different attine symbioses, were isolated from colonies for tests:

1) An *Acromyrmex octospinosus* fungus. The use of this fungus was deemed advantageous due to the following: first, sequencing data indicate that the strains cultivated by leaf-cutters (*Acromyrmex* and *Atta*) are phylogenetically very related and do not form discreet clusters by ant genus (Chapela et al. 1994; Bot et al. 2001; Silva-Pinhati et al. 2004); thus, by using an *Ac. octospinosus* fungus, the experimental *Atta* ants were exposed to a "leaf-cutter cultivar"; second, the use of such an *Acromyrmex* cultivar eliminates biases (ant chemical signals) found in fungal gardens taken from conspecific or congeneric *Atta* ants. *Atta* ants are very strongly territorial and aggressive, both intra- and interspecifically (personal observations). The specific chemical cues (pheromones) mediating fungus recognition are unknown; several cues have been proposed (Bot et al. 2001). The queen and workers could be potent sources of relevant pheromones. Thus a

fungus taken from an *Atta* spp. garden could elicit acceptance or rejection based on the ants' pheromones, not on the cultivar's own cues. In this work, choosing an *Acromyrmex* cultivar could have introduced *Acromyrmex* pheromones and biases, but these are expected to be less significant than *Atta* intra- or interspecific ones.

2) The second fungus tested was a *T. zeteki* cultivar, which was obtained from the same laboratory colony that donated the original cultivar used in the experimental, switched colonies.

The fungal symbionts were isolated in Petri dishes with moistened paper filter (to maintain high humidity) for 24 hours. Eggs and larvae were removed.

After these isolation periods for fungi and ants, workers were housed with cultivars in mini-nests (each housing 20-30 workers) placed in Fluon-coated trays, thus providing the ants with foraging arenas on the trays (see Fig. 3.1 and Chapter 3 for descriptions of trays and mini-nests). Six mini-nests were established for each cultivar type: three for *Atta* workers from native colonies and three for *Atta* workers from switched colonies. The cultivars were placed inside the mini-nests, on plastic weighing trays (3 cm diameter). Approximately 1 gram of fungus was provided, as well as oat flakes and fresh pear foliage (*Pyrus calleryana*) *ad libitum*.

Preferences were classified (and scored) as follows: **Rejection (1)**: ants actively carrying the fungus to a refuse pile, along with other debris; **Neutrality (2)**: ants ignoring the fungus, walking around and/or not contacting or not standing in continued contact (more than 30 minutes) with the fungus; **Arrestment (3)**: ants arrested, standing on the fungus consistently (longer than six hours at a time; observed every 30 minutes), but not adding substrate; **Cultivation (4)**: ants carefully incorporating processed plant substrate into the fungus garden in stereotypical cultivation behaviors. Observations were made daily for seven days.

To discriminate preference trends for cultivars, I compared the preference scores by means of the Mann-Whitney and Student's *t* tests (Sokal and Rohlf 1994; Lejarza and Lejarza 2004).

4.3 RESULTS

The switched colonies differed from the native colonies in several important and readily apparent aspects.

4.3.1 Appearance of Fungal Gardens.

Fungal garden color was very different between the *Trachymyrmex* and the *Atta* fungal symbionts (see Figure 3.1). The native (*Atta*) fungal symbiont showed an intense orange color. These gardens showed little aerial mycelium, the substrate particles were more or less clearly distinguishable to the eye, and the gongylidia were discrete. In these colonies, the ants deposited large amounts of fecal droplets around the entrances and in many areas of the nests.

In contrast, the *Trachymyrmex* symbiont cultivated by *Atta* showed a much paler pigmentation: whitish to tan color. The *Trachymyrmex* symbiont gardens were covered with aerial mycelium, which gave them a "fuzzy" appearance; and the clusters of gongylidia were hidden among this aerial mycelium and thus were less apparent. The outside of these colonies was relatively free of fecal droplets.

4.3.2 Changes in Queen and Fungus Garden Weights.

Values for queen and fungus garden weights are shown in Table 4.1. Table 4.1 shows queen and fungus garden weights in six colonies 128 days after establishment. At this date there were no significant differences in queen mass between native and switched colonies (Mann-Whitney test, $U = 8$, $Z = 1.5300$, $P = 0.1260$) although queens from native colonies were slightly heavier. On the other hand, there were significant differences in fungus garden mass between native and switched colonies (Mann-Whitney

test, U = 9, Z = 1.9600, P = 0.0250). Fungus garden weights after 228 days are shown in Figure 4.3. At this date there were also significant differences in fungus garden mass between native and switched colonies (Mann-Whitney test, Z = 1.7300, P = 0.04180).

4.3.3 Time Required by Colonies, from Inception, to Build a 423.5 cm^3 Fungus Garden in the Nest Boxes.

Two queenright *Atta* colonies cultivating an *Atta* fungal symbiont took 242 and 251 days (mean = 246) to fill their respective nest boxes (423.5 cm^3) with a fungus garden. The three colonies cultivating the *Trachymyrmex* symbiont took 316, 330, and 343 days (mean = 329.7) to fill their boxes. However there was no significant difference in this time requirement between native and switched colonies (Mann-Whitney test, Z = 1.7300, P = 0.0836). This trend (i.e. the switched symbiosis took 75% longer to fill the boxes) is nonetheless consistent with the above results on garden weight, where growth rate was faster for the native symbiosis.

4.3.4 Trends in Worker Number in Native and Switched Colonies of *Atta mexicana*.

Native *Atta* colonies: the fungal culture samples taken from the native colonies represented 5% of the respective fungal gardens. A total of 159 and 197 (average =178) workers were extracted from inside the 22.5-cm3 fungus samples from both colonies.

a) Smaller native *Atta* colony: for the smaller (16 month old) colony, maintained in 453.5 cm^3, the 178 workers/cm^3 average extrapolates to 2480 workers in the garden, besides the high worker concentration next to the brood, which was estimated to have 200 small workers, yielding a worker number of 2680. A total of 11 workers measuring more than 3 mm HW were also collected from inside and outside the nest box of this colony, for a minimum of 2691 workers for this smaller colony.

b) Older native *Atta* colony: This 28-month old colony had completely filled a nest box 18 x 14 x 11 cm (2772 cm^3). Thus, from the number of workers present in 22.5 cm^3 of

garden (178), this colony had at least 17,974 workers [(178) / 22.5 (2772)], without considering, for example, those smallest of workers clumped near the brood.

Symbiont-switched *Atta* colonies. These four 2001 and 2002 colonies produced only 200-400 workers each over the observation periods (16-28 months). It must be mentioned that all these switched colonies were housed in nest boxes of identical size to that of the 2002 native colony, which had at least 2691 workers. Both native and switched colonies had filled the boxes with fungus; thus they all had a very similar food amount (fungus garden volume) available for brood production. This is suggestive of a relation between symbiotic fungus type and numbers of workers produced.

4.3.5 Influence of Fungal Symbiont on Colony Developmental Pattern of *Atta mexicana,* and Comparison of Worker Size and Number Across Selected Attine Genera and Species

Table 4.2 summarizes HW size parameters for the experimental colonies reported herein, as well as from published reports on other attines.

***Trachymyrmex zeteki*.** The colony reported herein had 300-500 workers through three years. HW distribution of *T. zeteki* workers was very similar to that reported for *Trachymyrmex septentrionalis* (McCook) by Beshers and Traniello (1994); both species are considered monomorphic [although Beshers and Traniello (1994) consider *T. septentrionalis* slightly polymorphic; see Fig. 4.1 A and B]; they have a unimodal distribution with very narrow, rather symmetrical distribution curves. However *T. zeteki* workers are distinctly larger than those of *T. septentrionalis*; in this species the HW distribution mode is at 0.9 mm, whereas in *T. zeteki* the peak is at 1.3 mm. On the other hand *T. septentrionalis* has a much larger worker HW range (approx. 0.7-1.2 mm) (hence Beshers and Traniello (1994) conclusion of slight polymorphism) versus 1.2-1.32 mm for *T. zeteki*. (Fig. 4.1A and B).

***Atta* spp.** There are no published reports on worker size distribution for *A. mexicana*. I generated the worker size distribution for the 28-month old native colony, an ergonomic

phase colony (Fig. 4.1 E). These data can be compared to the size distribution in ergonomic and mature colonies of several species of *Atta*, *Acromyrmex,* and *Trachymyrmex* (Fowler 1983; Wilson 1980, 1983a, 1983b; Wetterer 1999) (Fig. 4.1 A-H).Native, ergonomic stage colonies of *Atta sexdens*, *A. texana,* and *A. mexicana* (this work) show similar proportions of worker sizes, with a peak in the proportion of workers with a HW less than 1 mm, and a long, low "tail" surpassing 4 mm (soldiers) (fig. 4.1 C, D, E). As mentioned, these larger individuals represent most of the worker biomass in colonies (Wetterer 1999).

Native and switched colonies. Sharp differences in worker size and numbers were observed between workers from *A. mexicana* colonies cultivating the different symbionts. Switched colonies produced workers of a restricted size range. The worker size distribution is shown for the 2001, 28-month old switched colony (Fig 4.1 F).

It must be emphasized that in laboratory colonies across *Atta* species (*A. cephalotes, A. colombica, A. sexdens*) the ergonomic phase and soldier production begin after 5-7 months or so (Weber 1972; Sánchez-Peña, unpublished observations); soldiers (≈ 4 mm HW) are the last of the worker size categories or castes to appear. In this work, after 16 months, the three 2002 switched colonies produced a range of 300-400 workers, most with a HW smaller than 1 mm. The largest worker sizes produced in each of these colonies (in HW) were 2.6, 1.3, and 1.7 mm. Their worker size range approached that of the 2001 switched colony (with a largest worker HW= 2.0 mm) (Fig. 4.1 F). The dissimilarities are more apparent when comparing the maximum worker size of fungus-switched colonies (maximum HW=2.6) with ergonomic-phase *Atta* spp. colonies (*A. sexdens* HW= 3.9; *A. texana* HW=5.5; *A. mexicana* [this work] HW=5.1 and 4.1; *A. mexicana* (mature field colony in Jalisco, Mexico) HW=4.2) (Table 4.2) (Fowler 1983; Wilson 1983a; A. San Juan and H. Li, personal communication).

In general, the worker size production in switched colonies was much skewed (>90%) towards workers smaller than 1 mm HW. In *Atta* spp. this proportion ranges around 50% for colonies in the ergonomic and reproductive phases (both phases being more than

several months old, and having above a few thousands of workers); minima worker size (HW < 1.2 mm) constituted 48% of a large *Atta cephalotes* colony (Stradling 1978). In this work, the native 28-month old colony of *Atta mexicana* had 72 % of its workers with a HW< 1.2 mm; for the switched colony, these where 87 %. Media workers (HW 1.2-3.2 mm) also constitute a significant proportion, about 40%, of ergonomic stage-colonies across *Atta* species (*A. cephalotes*, *A. texana*, *A. sexdens*) (Wilson 1980, 1983a, 1983b; Fowler 1983; Wetterer 1999). In this work, the native 28-month old colony of *Atta mexicana* had 28% of its workers with a HW> 1.2 mm; for the switched colony, these where only 13 %. While these data differences might be due in part to species differences in worker size distribution, they also show that the native *A. mexicana* colony was more similar to the mature colonies of other *Atta* species than to the switched colony, particularly regarding workers larger than 1.2 mm HW (28 vs. 13%, respectively).

There was a drastic contrast in worker size between symbiont-switched colonies and the native colonies of *A. mexicana* (all chronologically "mature" in this respect). The switched colonies lacked the distribution "tail" encompassing the largest worker sizes of native colonies. Comparisons can be made with published data on worker size for other attines (Fig. 4.1). Wetterer (1999) considered that young and mature *Acromyrmex coronatus* (F.) colonies had worker HW distributions similar to those of the transient distributions of small, pre-ergonomic (26-486 workers) *Atta cephalotes* colonies (Wilson 1983b) and "only slightly broader than the *T. septentrionalis* colony" (HW ranges = 0.7-1.2 mm and. 0.6-1.7 mm for *T. septentrionalis* and *Ac. coronatus* respectively). Wetterer (1999) argued that the restricted size range of *Ac. coronatus* emphasizes the basal, transitional character of this species among the higher attines regarding worker size.

4.3.6 Worker Acceptance of Native and Novel Cultivars.

Table 4.3 summarizes worker acceptance of *A. mexicana* workers reared with *Atta* (leaf-cutter) or *Trachymyrmex* symbiotic fungi, when given a choice between a leaf-cutter (*Acromyrmex octospinosus*) and a *Trachymyrmex* cultivar. When considering as main factor the type of maternal colony, *Atta mexicana* workers raised on an *Atta* fungus

(native colony) chose, without exception and unambiguously, the leaf-cutter cultivar over the *Trachymyrmex* cultivar. For these workers, there were significant differences in preference for different cultivars (Student's *t* test values: $t = -6.71$, df = 6, p = 0.01; Mann-Whitney test: U = 36, Z = 2.8800, P = 0.0040). In comparison, the reactions of the *Atta* workers raised on a *Trachymyrmex* symbiont (switched colony) can be described as equivocal. For these workers, there were no significant differences in cultivar preference ($t = -1.28$, df = 5, p = 0.256; Mann-Whitney's U = 26, Z = 1.2800, P> = 0.2005). In two (one third) of the replicates, the switched-colony workers accepted the leaf-cutter cultivar. In the remaining replicates the reactions included arrestment, neutrality, and rejection.

When considering as main factor the cultivars tested for acceptance, there were highly significant differences in *Trachymyrmex* cultivar acceptance between workers reared on either native or symbiont-switched colonies. *Atta* workers reared on *Trachymyrmex* fungus were less rejecting of the *Trachymyrmex* cultivar, while *Atta* workers reared on *Atta* fungus rejected it at all times ($t = -3.16$, df = 5, p = 0.025). There were no significant differences in acceptance of a leaf-cutter cultivar by *Atta* workers reared on either native or symbiont switched colonies; both accepted this cultivar to a similar degree ($t = 0.696$, df = 5, p = 0.518; Mann-Whitney's U = 13.5, Z = 0.7200, P> = 0.4715).

4.4 DISCUSSION

4.4.1 Pigmentation of Fungus Gardens, Weight Changes, and Growth Rates.

As shown in Figure 3.1, the fungus gardens of the switched symbiosis were much paler than the native symbiosis; these showed a reddish pigmentation. Personal observations indicate that when these symbionts are cultivated in liquid media like Potato Dextrose Broth, their appearance is the opposite: *Trachymyrmex* symbionts produce dark pigments; while the cultures of *Atta* and *Acromyrmex* fungi are relatively free of pigments.

Queen weight changes and fungus growth rates (in weight and volume) point towards rapid negative effects for both symbionts in the novel association. Although not

statistically significant, queens weighed less when living in the switched symbioses. Fungus growth gains in weight and volume were also always smaller in the novel association than in the native symbiosis, although only weight gains were significantly different. These trends *per se* would imply negative consequences for fungus-switched colonies as a whole.

4.4.2 Worker Number and Size Distribution in Native and Switched *A. mexicana* Colonies.

After 16 months, the 2002 colonies of *A. mexicana* cultivating their native fungal symbiont produced many times more workers than equally aged and older colonies cultivating a novel symbiont, that of *T. zeteki*; both colony types had a nearly identical volume of fungus garden available (same nest box size) (Fig. 3.1). Switched colonies never produced soldiers. See Table 4.2 for a comparison of mean and median HW of ant colonies referred to in this work. The results reported herein further emphasize the biological differences between the fungal symbionts

Currie et al (2003) emphasized the differences between the members of the higher attines. They summarize information and split the higher attines between the "*Trachymyrmex* symbiosis" clade, including the genera *Trachymyrmex* and *Sericomyrmex*, and the "leaf-cutter symbiosis" clade, which includes *Atta* and *Acromyrmex*. The major traits dissecting the higher attines among these clades are the biological traits and phylogenies of the ant, symbiotic fungus, and parasite (*Escovopsis*). Ant phylogenies indicate a more basal position for *Trachymyrmex* relative to the leaf-cutters *Atta* and *Acromyrmex* (Chapela et al 1994; Schultz and Meier 1995; Wetterer et al. 1998). This basal position is also reflected in the phylogeny of the specialized parasite, *Escovopsis*; the *Trachymyrmex*-associated strains are basal respective to those associated with the leaf-cutters (Currie et al. 2003). The data from fungal symbionts indicate that both the *Trachymyrmex* and the leaf-cutter fungi are highly derived. However, considering the relative phylogenetic positions of the ants and *Escovopsis* parasite associates (Chapela et al 1994; Mueller et al. 1998; Currie et al. 2003), it is not unreasonable to consider that the *Trachymyrmex*

symbiotic fungi are probably less derived. These reports also suggest that the lineage giving rise to the leaf-cutter fungal symbionts probably originated from an ancestral state that maybe largely retained in the extant *Trachymyrmex* symbiosis (Weber 1972, Mueller et al. 1998).

Extreme polymorphism and, in particular, the presence of a specialized defense caste (the soldiers) are the landmarks of the genus *Atta*. Associated with this polymorphism are extensive divisions of labor (Weber 1972, Wetterer 1990). Workers of different size ranges perform different tasks, such as brood care, digging, foraging, and defense. At the nest, the smallest workers prepare the material retrieved by larger workers as fungal substrate. Wetterer (1999) suggested that both the extreme polymorphism of *Atta* and the nearly bimodal, polymorphic worker size of *Acromyrmex* species like *Ac. octospinosus, Ac. echinatior*, and *Acromyrmex volcanus* originated independently from an ancestor that exhibited a narrow polymorphism like *Ac. coronatus* (worker size range of 0.6-1.7 mm HW), or an even more limited "polymorphism", similar to that of some advanced *Trachymyrmex* species like *T. septentrionalis* (0.7-1.2 mm HW). This is relevant in connection with the present work because all four *Atta mexicana* colonies (three 16-months old and one 28-months old) cultivating the *Trachymyrmex* fungi produced worker numbers (300-400) which are similar to those of *Trachymyrmex* spp., which probably more closely resembles the ancestral state. The 28-month old switched colony also showed a worker size distribution and size range very similar to that of *Ac. coronatus* (Figure 4.1 F, G, H; Table 4.2 F-H) (Weber 1972; Hölldobler and Wilson 1990; Wetterer 1990; Beshers and Traniello 1994; Schultz and Meier 1995; Wetterer 1999). Very young, native *Atta* colonies show a restricted (but rather different) size distribution and size range (Wilson 1983b), but this is ephemeral: the colonies grow very rapidly (exponentially) entering the ergonomic phase within weeks (Hölldobler and Wilson 1990), produce soldiers within 5 to 7 months in the laboratory and produce 250,000-300,000 workers after 24 months in nature (Weber 1972). As colonies expand in size, they expand their worker size range (Weber 1972, Wetterer 1990, 1999). In contrast, during the 16-28 month period, the ant number in the four switched colonies in this work

grew only to 200-400 workers, versus >2600 and >17,000 workers for laboratory colonies cultivating their native fungus. It must be emphasized again that all switched colonies and the 2002 native colony (which had >2600 workers) were housed in nest boxes of identical size and were "fed" with identical plant material. Thus all of them had very similar potential to grow their fungus gardens and indeed all ended up filling the identical nest boxes with fungus (Fig. 4.1), but the switched colonies produced much smaller and fewer workers.

It has been speculated that the allegedly advanced and more efficient fungal strains of the leaf-cutters allowed them to attain their phenomenal colony size and great ecological success (Stradling and Powell 1986). Alternatively it can be proposed, however, that these advanced traits (polyphagous herbivory, extreme polymorphism and very large colony size) have been driven by behavioral and morphological modifications on the ants' side –without significant modifications in their fungal strains; these strains would remain physiologically/ functionally similar to those in the ancestral, *Trachymyrmex*-like symbiosis. This report points towards a high level of functional coadaptation between fungus and ants in the leaf-cutter symbiosis. It also lends support to the idea that the fungal cultivar has been crucial for the evolution of herbivory from detritovory, as well as polymorphism and very large colony sizes of the leaf-cutting Attini. However, in these observations is difficult to be conclusive about fungal main effects since these observations measured ant-fungus interaction and fungus as main effect together.

In this work, the apparent similarity in worker numbers and size range between the "old", switched *Atta* colonies and relatively basal species like *T. zeteki*, *T. septentrionalis* and especially *Ac. coronatus* is intriguing, and I consider it close to being a fungal symbiont-induced evolutionary regression readily visible on the ant's side. I propose that the severely restricted development in the switched colonies is directly due to the cultivation of a primitive symbiont, and is indicative of the probable colony condition found in the ancestor of the leaf-cutter symbiosis.

4.4.3 Worker Preference for Native (Native) and Novel Cultivars: Innate and Learned Components of Cultivar Acceptance.

The observations reported in this Chapter and Chapter 3 showed that the type of symbiotic fungus used as larval food had little effect upon acceptance of fungus by the resulting naïve adults. On the other hand, the cultivar that these naïve workers accepted as their own influenced their subsequent acceptance of different cultivars.

In several insect models, larval food appears to affect subsequent adult food preference, a phenomenon considered as larval learning. However, comparisons of ants with other insects regarding the effect of larval food upon larval learning and adult behavior should be limited. For example, in ants, adult workers and larvae usually feed on the same food items, although adults feed on liquefied food only as opposed to particulate food in larvae (Glancey et al. 1981; Eisner and Happ 1962), while in most other holometabolous insects, larval and adult foods are very different. In these insects, larval food could affect adult behaviors like selection of oviposition substrates, a phenomenon not comparable in ants. Ants often retrieve and process food items collectively, and interactions between workers of different ages possibly reared on different food items are unavoidable. This implies that individual choice is restricted. Therefore the effects of larval learning and food preference in adults can be compared between ants and other insects in a general way, but the mechanisms and ultimate factors selecting for larval learning can be very different.

There is evidence for retention of larval learning through metamorphosis in insects, including ants (Isingrini et al. 1985; Carlin 1988; Carlin and Schwartz 1989), *Drosophila* (Tully et al. 1994) and the mealworm *Tenebrio* (Alloway 1972; Punzo and Malatesta 1988). In his review of brood recognition in ants, Carlin (1988) suggested that learning of environmental recognition cues by larvae could influence inter-colony recognition of brood as adults. For example, Isingrini et al. (1985) demonstrated pre-imaginal learning in the ant *Catagliphis cursor*. They transferred eggs from their natal colony to alien colonies. When larvae hatching from these eggs became pupae, they were returned to their natal nest, and tested for brood preferences when they became adults. These adults

preferred larvae from the colony in which they spent their larval life over larvae from the colony they were born in, i.e. preference of unrelated larvae over their immediate kin. Carlin and Schwartz (1989) also provided evidence that learning can begin at the larval stage in *Camponotus.* Studies with *Drosophila* have given varied (usually negative) results (Barron and Corbet 1999). Tully et al. (1994) tested the memory of *D. melanogaster* adults who had been trained as larvae before metamorphosis; they show that larval *D. melanogaster* can learn to avoid an odor that is paired with presentation of an electric shock. The conditioned odor avoidance in larvae can still be detected in the adult flies eight days later. These observations suggest that, unlike most other tissues, significant memory-related aspects of the *Drosophila* central nervous system maintain their integrity and connections through metamorphosis after the third larval instar. The experiments of Tully et al. (1994) are convincing, but Barron and Corbet (1999) criticized the use of the electric shock stimulus as irrelevant to the natural situation. In the holometabolous insect *Tenebrio obscurus*, Punzo and Malatesta (1988) studied brain RNA synthesis associated with transmission of larval learning into the adult. Learning was accompanied by an increase in RNA synthesis within the corpora pedunculata of both larval and adult stages of *T. obscurus*. Beetles were able to retain larval learning of a complex maze task through metamorphosis. It was proposed that neural organization is conserved through metamorphosis within those brain regions associated with the consolidation and storage of experiential information.

In general, the possibility of larval learning is surprising. Holometabolous insects show a dramatic and complete metamorphosis between a distinct larval stage and a distinct adult stage, separated by a pupal stage. During the pupal stage of *Drosophila* (and likely also in most holometabolous insects) the larval sense organs undergo histolysis and adult sense organs are formed *de novo* from imaginal disks (Carlson 1991). Other radical changes in the nervous system are associated with metamorphosis. The mushroom bodies (the 'memory centres' of the insect brain) are extensively neuronally rewired during metamorphosis (Wigglesworth 1984; Carlson 1991). Given these dramatic changes in the structure of the nervous system, any demonstration that an adult insect retains learning

acquired in the larval stage would be of great interest. Some of the earlier published evidence of larval learning in *Drosophila* (i. e. Thorpe 1939) might require reassessment, since the proposed larval learning in this case is likely due to the influence of puparial contaminants from the larval environment on the behavior of adult flies (Barron and Corbet 1999). These observations by Thorpe (1939) are also difficult to interpret because he used raw mixtures containing several compounds. From their experiments and review, Barron and Corbet (1999, 2000) concluded, "There is no evidence that a change in behavior of adult *Drosophila* can be induced by exposure to a conditioning stimulus restricted to the larval stage. We found no evidence for preimaginal conditioning. Therefore, exposure during the adult stage is more important than larval exposure in shaping adult behavior in *Drosophila*." Thus, consistent with these previous findings, natal colony and symbiotic fungus did not seem to have a major effect on nestmate and cultivar acceptance by naïve *Atta* workers in this work.

The preference patterns reported in this chapter indicate that the process whereby workers adopt a fungal strain as a permanent symbiotic cultivar has at least two components: there is an innate component and, perhaps more importantly in the higher attines, a learned component that can partially override at least some innate predispositions of workers. I showed both the permanent adoption of novel symbiotic fungi by full colonies, and also the equivocal acceptance (in switched workers) towards an otherwise acceptable leaf-cutter cultivar. When testing cultivar acceptance in workers from native and switched colonies, I must mention that in order to avoid recognition of their own or congeneric chemical cues, I sought to devise a more rigorous experiment. Therefore I tested the ants' preference using fungi taken not from their native colonies, but from a *Trachymyrmex* and an *Acromyrmex* colony (see Material and Methods).

Concerning colony environment and adult cultivar acceptance, my observations on the consistent and universal rejection of *Trachymyrmex* cultivars by "experienced" *Atta* workers, indicate that there is a temporary period of sensitivity shortly after adult worker eclosion from the pupa, when assimilation of cultivar and nestmate signals occurs. The results of Chapter 3 also support this conclusion (i.e. adult, naïve workers of *Atta* and

Trachymyrmex raised on their native fungi as larvae, permanently accepted novel symbionts).

In Chapter 3, I found additional indications of the low influence of larval environment upon subsequent adult behavior (in this case, coworker acceptance) in the genus *Atta*: I took pupae from different colonies belonging to different *Atta* species (*A. cephalotes* and *A. mexicana*). These pupae originated naïve workers that coexisted peacefully and cooperated with workers of the other species in mixed nests.

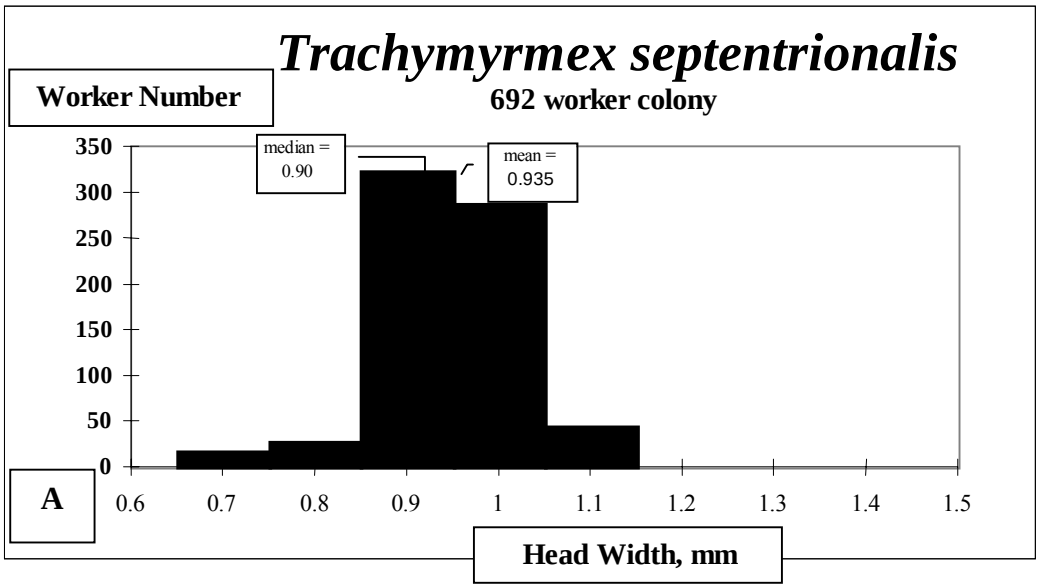
Imprinting is a specialized, rapid learning process limited to a transient period ("sensitive period" or "critical period") usually early in the life of an animal, and establishes a behavior pattern (such as recognition and attraction to its own kind or a substitute). It also appears to be irreversible. The process of imprinting is genetically determined, but the particular object to be imprinted on is learned (Lorenz 1981; Dejean 1990). The concept has been developed mainly from research on birds and mammals, and there are many variations of behaviors classified as imprinting across animal taxa.

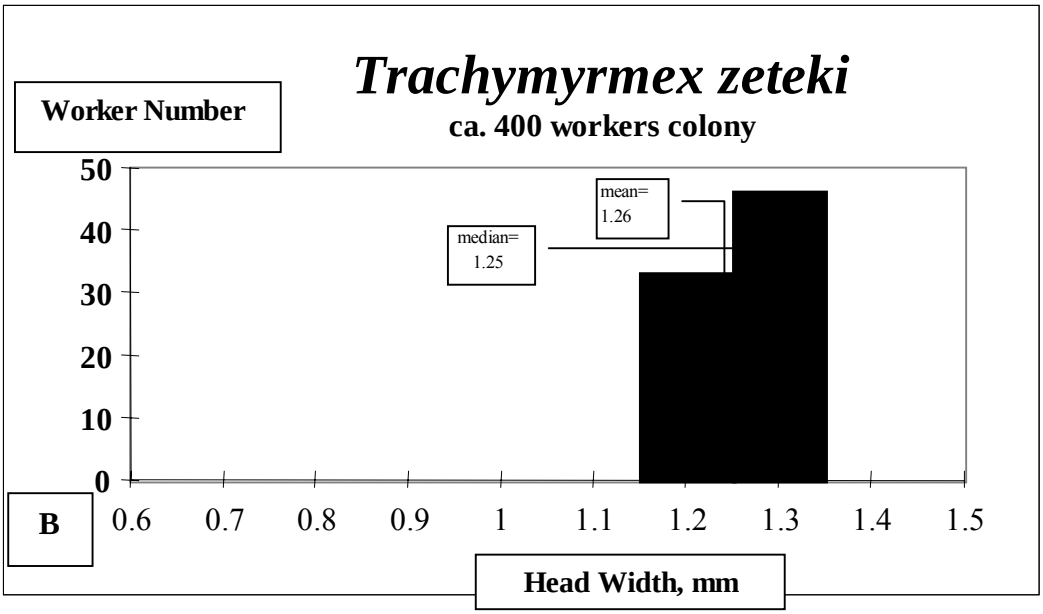
My observations show that the interaction between *Atta* ants and their *native* fungal symbionts resembles the classic definition of imprinting: early after adult eclosion, ants adopt their symbiotic fungi, and these ants exposed to the native fungus will never accept a non-leafcutter cultivar, even in non-choice tests where this rejection results in the ants' death by starvation. The acceptance and cultivation of the novel (switched) fungus in the switched colonies resemble imprinting in a general way, but their subsequent acceptance of a leaf-cutting cultivar indicate that an innate component is present and can possibly override cultivar imprinting-like phenomena. On the other hand, in these experiments the ants were not provided with a choice of cultivars (i.e., novel versus native), so the irreversibility of the ant's attachment to a novel cultivar remains to be tested.

The rapid and full acceptance of the *Acromyrmex* cultivar by *Atta* workers reared on *Atta* fungus confirms the similarity between *Atta* and *Acromyrmex* cultivars suggested by phylogenetic information (Bot et al 2001; Silva-Pinhati et al. 2004; S. Rehner personal

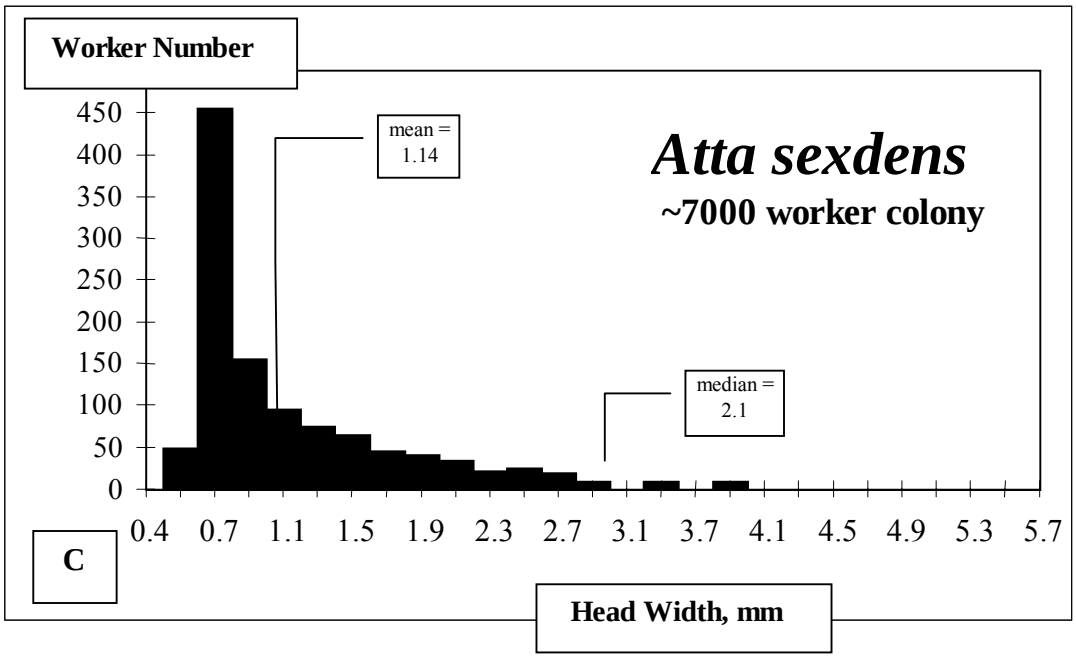
communication); the genetic closeness of these fungi appears reflected as sensorial likeness towards the ants.

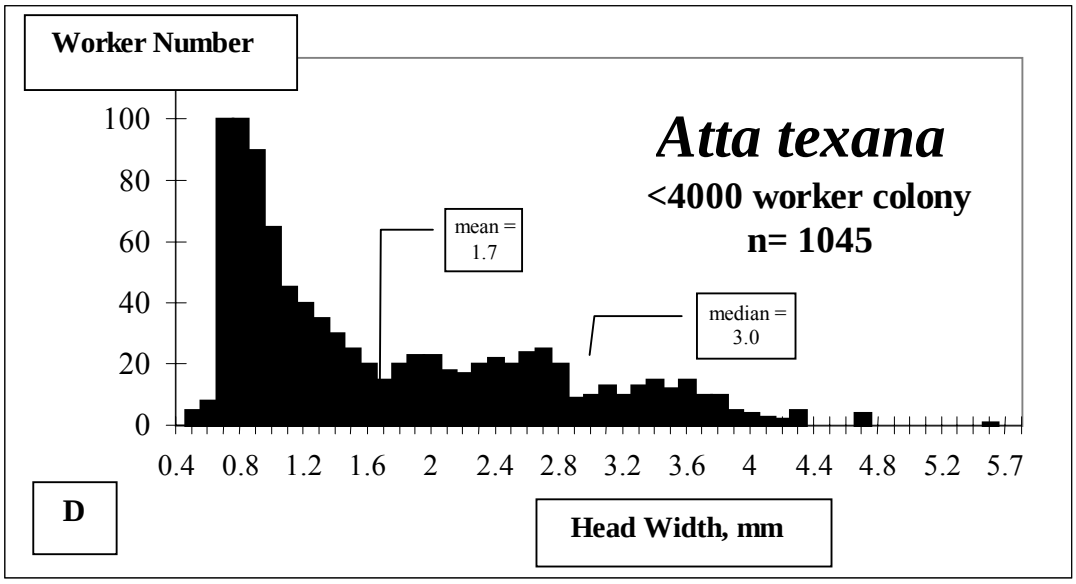
Figure 4.1A-H. Distribution of head width (mm) for workers of selected attine species. **C-H.** Head width distribution for workers of ergonomic-to-mature stage colonies of leaf-cutting ants: *Atta* spp. and *Acromyrmex coronatus*. **A.** *Trachymyrmex septentrionalis* from USA (from Beshers and Traniello, 1994). **B.** *Trachymyrmex zeteki* from Panama.



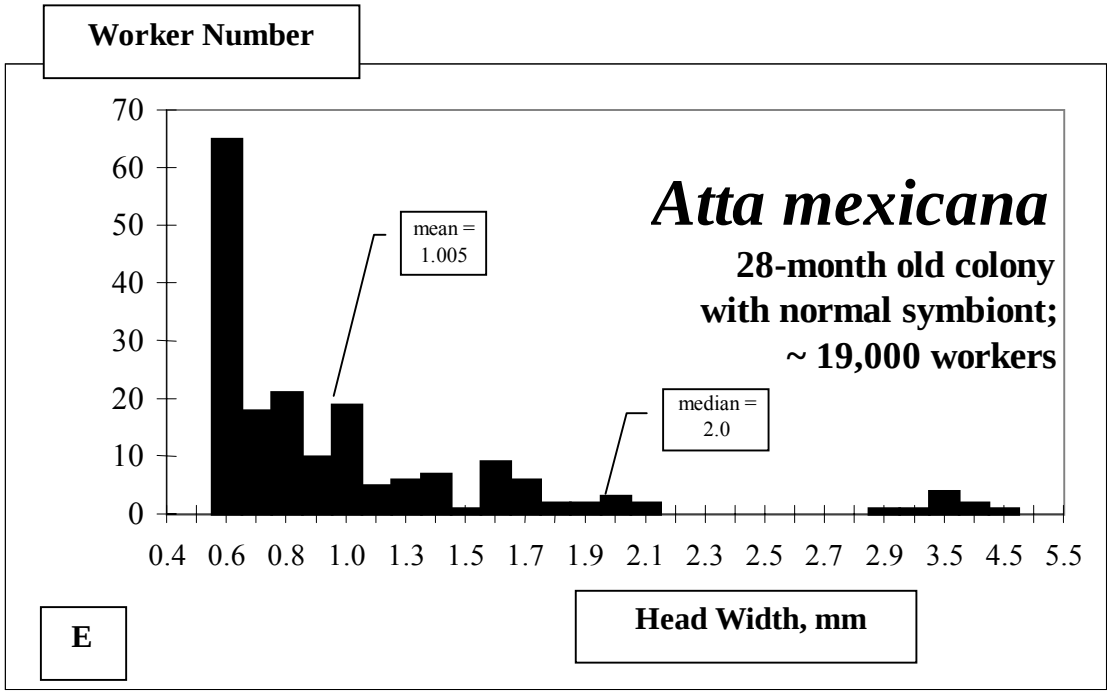


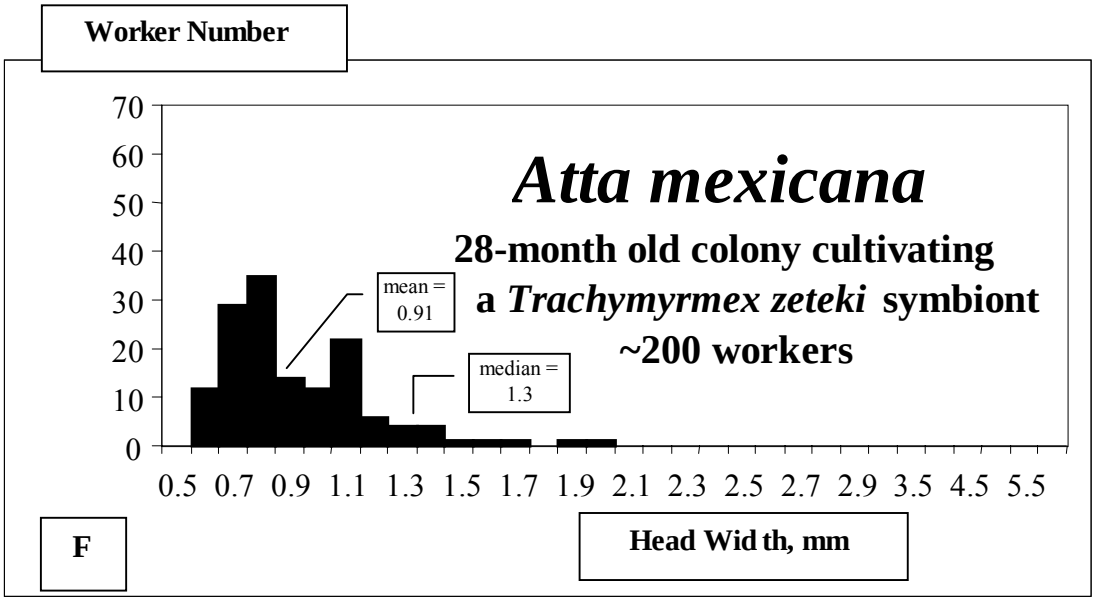
4.1 continued. C. *Atta sexdens*, ≈ 7000 workers (Wilson 1981 in Wetterer 1999). **D.** *Atta texana* laboratory colony, ≈ 4000 workers (Fowler 1983 in Wetterer 1999).





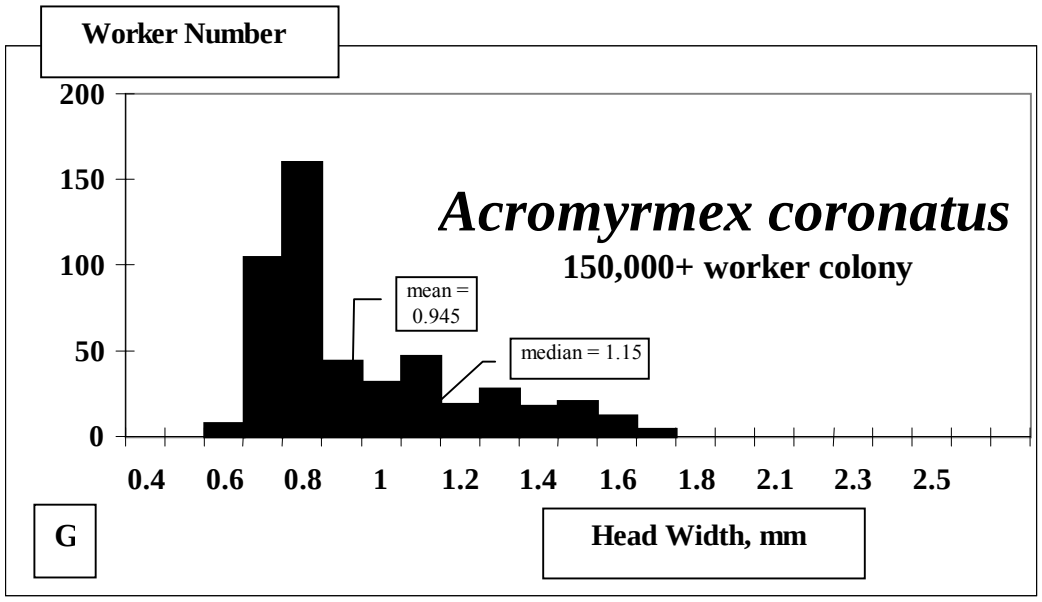
4.1 continued. 28-month old laboratory colonies of *Atta mexicana*. **E.** Colony cultivating its native fungal symbiont; total worker number 18,000-19,000. **F.** Colony cultivating a *Trachymyrmex zeteki* fungal symbiont; total worker number about 200.





4.1 continued. G. *Acromyrmex coronatus*, 150,000+ workers; (Wetterer 1999).

H. *Acromyrmex coronatus*, 11,922 workers (Wetterer 1999).



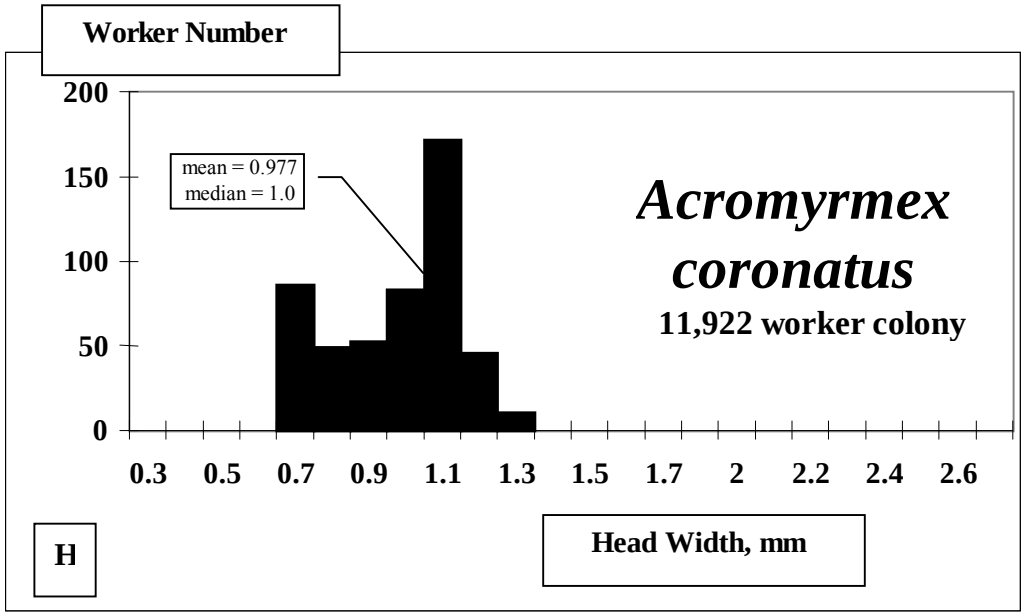
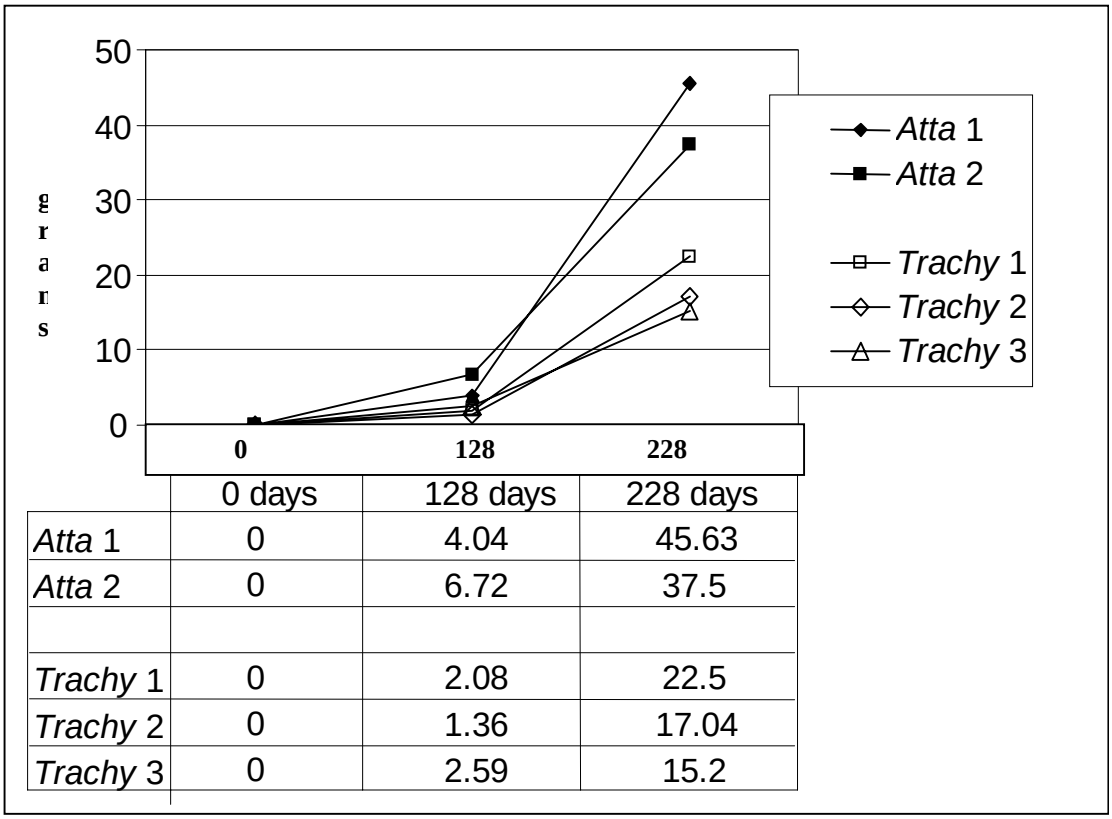


Figure 4.2. Fungus garden weight (grams) in six *A. mexicana* colonies 128 and 228 days after establishment.

Colonies cultivated either their native *Atta* fungus or a *Trachymyrmex* symbiont. There were significant differences in fungus garden mass between native and switched colonies after 128 and 228 days (Mann-Whitney test, U = 9, Z = -1.9600, P = 0.0250).



	0 days	128 days	228 days
Atta 1	0	4.04	45.63
Atta 2	0	6.72	37.5
Trachy 1	0	2.08	22.5
Trachy 2	0	1.36	17.04
Trachy 3	0	2.59	15.2

Table 4.1. Queen and fungus garden weight in six *A. mexicana* colonies 128 days after establishment.

Fungal symbiont and colony number	Fungus mass in milligrams	Queen mass in milligrams
Trachymyrmex 1 (T 1)	2082.4	167.8
Trachymyrmex 2 (T 2)	1369.4	151.8
Trachymyrmex 2 (T 3)	2594.4	158.6
Average *Trachymyrmex* fungus	2015**a**	158.6**a**
Atta 1	4041.8	187.8
Atta 2	6723.2	172.5
Atta 3	3936.1	161.2
Average *Atta* fungus	4900.33**b**	173.8**a**

Colonies cultivated either their native *Atta* fungus or a *Trachymyrmex* symbiont. There were no significant differences in queen mass between native and switched colonies (Mann-Whitney test, U = 8, Z = 1.5300, P = 0.1260); there were significant differences (indicated by different lowercase letters) in fungus garden mass between native and switched colonies (Mann-Whitney test, U = 9, Z = -1.9600, P = 0.0250).

Table 4.2. Worker size (head width, in mm) for selected higher attine species. Unless otherwise indicated, all listed colonies had their native symbiotic fungus. All these native-fungus colonies are mature regarding the size of workers produced (with the possible exception of the smaller *Acromyrmex coronatus* colony).

Ant species	Mean HW (mm)	Median HW (mm)	Maximum worker size
Trachymyrmex septentrionalis (1)	0.935	0.90	1.15
Trachymyrmex zeteki (2)	1.26	1.25	1.35
Acromyrmex coronatus 11,922 workers (3)	0.97	1.0	1.3
Acromyrmex coronatus 150,000+ workers (3)	0.94	1.15	1.7
Atta texana (4)	1.7	3.0	5.5
Atta sexdens (5)	1.14	2.1	3.9
Atta mexicana native (6)	NT	NT	4.2
Atta mexicana native 1, 2001 (2)	1.0	2.0	5.1
Atta mexicana switched* 1, 2001 (2)	0.91	1.3	2.0
Atta mexicana switched* 2, 2002 (2)	NT	NT	2.6
Atta mexicana switched* 3, 2002 (2)	NT	NT	1.3
Atta mexicana switched* 4, 2002 (2)	NT	NT	1.7

(1)= Beshers and Traniello 1994; (2)= this work; (3)= Wetterer 1999; (4)= Fowler 1983; (5)= Wilson 1983a; (6)= San Juan and Li personal communication; NT= data not taken; * = colonies switched to a *Trachymyrmex zeteki* fungus.

Table 4.3. *Atta mexicana* worker preference for native and novel cultivars. "Fungus on which workers were raised (replicates)" (A1-6, T1-6) indicates whether workers had an *Atta* (A) or a *Trachymyrmex* (T) fungus as cultivar in their maternal colony.

Fungus on which workers were raised (replicates)	**Tested Cultivar, Preference and Preference Rank**	
	Trachymyrmex zeteki	**Leaf-cutter (*Acromyrmex octospinosus*)**
Atta 1	**R (1)**	**C (4)**
Atta 2	**R (1)**	**A (3)**
Atta 3	**R (1)**	**C (4)**
Atta 4	**R (1)**	**A (3)**
Atta 5	**R (1)**	**C (4)**
Atta 6	**R (1)**	**C (4)**
	Trachymyrmex zeteki	**Leaf-cutter (*Acromyrmex octospinosus*)**
Trachy 1	**R (1)**	**C (4)**
Trachy 2	**A (3)**	**N then A (2.5)**
Trachy 3	**A (3)**	**A then R (2)**
Trachy 4	**A (3)**	**A (3)**
Trachy 5	**R (1)**	**C (4)**
Trachy 6	**A (3)**	**N then A (2.5)**

Table 4.3 legend continued on next page.

Table 4.3 continued. Preference was classified and scored as follows: Rejection **(R) (score=1)**, ants actively carrying the fungus to a refuse pile, along with other debris; Neutrality **(N) (score=2)**, ants ignoring the fungus, walking around and/or not contacting or not standing in continued contact (more than 30 minutes) with the fungus; Arrestment **(A) (score=3)**, ants arrested, standing on the fungus consistently, after six hours; Cultivation **(C) (score=4)**, ants carefully incorporating plant substrate into the fungus in the stereotypical cultivation behaviors. For workers of the native-fungus colony, there were significant differences in preference for different cultivars (Student's *t* test values: $t = -6.71$, df = 6, $p = 0.01$; Mann-Whitney test: U = 36, Z = 2.8800, P = 0.0040). In comparison, for workers of the switched-fungus colony (raised on a *Trachymyrmex* fungus) there were not significant differences in cultivar preference ($t = -1.28$, df = 5, $p = 0.256$; Mann-Whitney's U = 26, Z = 1.2800, P > = 0.2005). In two (one third) of the replicates the switched-colony workers chose the leaf-cutter cultivar. In the remaining replicates the reactions included arrestment, neutrality, and rejection.

When considering as main factors the cultivars provided for choice, there were highly significant differences in *Trachymyrmex* cultivar acceptance between workers reared on either native or symbiont-switched colonies. *Trachymyrmex* fungus-reared workers were less rejecting of the novel cultivar, while *Atta* fungus-reared workers rejected it at all times ($t = -3.16$, df = 5, $p = 0.025$). There were no significant differences in *A. octospinosus* cultivar acceptance for workers reared on either native or symbiont switched colonies; both accepted this cultivar to a similar degree ($t = 0.696$, df = 5, $p = 0.518$; Mann-Whitney's U = 13.5, Z = 0.7200, P > = 0.4715).

Chapter 5: Tetratrophic Systems in Fungus-Growing Ants: Susceptibility to Infectious Disease in *Atta mexicana* Workers Raised on a Native (*Atta*) or a Novel (*Trachymyrmex*) Cultivar

Synopsis. *Atta* colonies can have millions of workers (Weber 1972). For larval nutrition and development, these ants depend completely on the cultivation of specialized strains of basidiomycetous symbiotic fungi in the Lepiotaceae (Chapela et al. 1994; Mueller et al. 1998). There is a paucity of empirical data on the suspected specific coadaptations, of any kind, between leaf-cutting ants and their highly derived symbionts. My observations (Chapter 4) indicated that *Atta mexicana* workers from colonies cultivating a novel symbiotic fungus (from *Trachymyrmex zeteki*) have a restricted worker size distribution, which lies in the smaller size range of that found in non-switched colonies of similar age. Nonetheless, it remains unknown whether development of larvae on this novel symbiont has negative effects on the physiological condition of the workers. I studied these possible effects by testing the susceptibility to infectious disease (*Beauveria bassiana* mycosis) of *Atta mexicana* workers raised to adulthood on either an *Atta mexicana* or a *Trachymyrmex zeteki* fungal symbiont. The experimental challenge with this insect pathogen indicated that workers raised on colonies cultivating the novel symbiont were no more susceptible to infections by *B. bassiana* than workers raised on the native symbiotic fungus. The fungal switch thus did not seemed to affect ants regarding sensitivity to the pathogen; they appeared physiologically as competent in this respect as workers from colonies cultivating the native fungal symbiont. It is possible that the developmental "stunting" (restricted worker size range) induced by the non-native symbiotic fungus is not due to simple starvation (which would probably have produced stressed, weaker, and more susceptible ants) but instead due to altered regulation of development, which resulted in ants of restricted size but otherwise competent organisms.

5.1 INTRODUCTION

The New World leaf-cutting ants of the genus *Atta* are the most derived of the fungus-growing ants of the tribe Attini (Hymenoptera: Formicidae: Myrmicinae). *Atta* colonies can have millions of workers (Weber 1972). These ants are completely dependent, for larval nutrition and development, on the cultivation of specialized strains of basidiomycetous symbiotic fungi (Agaricales: Lepiotaceae) (Chapela et al. 1994, Mueller et al. 1998). It has been estimated that the subterranean fungus gardens of *Atta* colonies can weigh almost 150 kg (Stahel 1943), and individual *Atta* colonies can produce almost 5 kg of ants (Weber 1972). These tremendous outputs are assumed to have selected for fungal strains that are very highly efficient at biomass transformation, both from plants to fungus, as well as from fungus to ants.

Few data exist on the suspected specific coadaptations between leaf-cutting ants and their highly derived fungal symbionts. The related, non-leaf-cutting ants of the genera *Trachymyrmex* and *Sericomyrmex* (herein collectively referred to as the *Trachymyrmex* symbiosis) cultivate symbionts that are related but distinct, and less derived, than the leaf-cutting symbiotic fungi (Currie et al. 2003). No switches of cultivars between these groups have ever been reported from natural colonies in the field, despite extensive sampling (Chapela et al. 1994; Bot et al., 2001; S. Rehner, personal communication) and despite ample opportunities for such switches in nature, given the biology, abundance and extensive sympatry of both groups. For example, *Trachymyrmex* colonies commonly nest and forage on *Atta* mounds (Weber 1972; personal observations). Stradling and Powell (1986) mention, in an anecdotal way, that one colony of *Trachymyrmex zeteki* Weber flourished after their own decaying fungal garden was substituted for an *Atta cephalotes* (L.) fungus. However their report provides no additional detailed or quantitative data on this single observation. My observations show that *Atta* will accept and cultivate a *Trachymyrmex* fungal symbiont, but this will have radical negative consequences, at the colony level, for both symbionts (Chapter 4). It is unknown whether development of larvae on a novel symbiont will have negative effects on the health and disease resistance condition of individual workers.

Infectious diseases by fungi related to *B. bassiana* (Ascomycota: Clavicipitales) are seemingly major mortality factors on some ant populations in the tropics (Evans 1982). Related clavicipitalean infections have been reported in *Acromyrmex octospinosus* Reich in Panama (van Borm et al. 2002). Entomopathogenic fungi have often been reported as causes of lethal infections of tropical and subtropical leaf-cutting ants: examples are *Cordyceps* sp. on *Atta* spp. in Amazonian rain forests (Evans 1982); *B. bassiana* on *A. mexicana*, on agricultural zones of Sinaloa, Mexico (Sánchez-Peña 1990) and in forested habitats of NE Mexico (Chapter 2); *B. bassiana* on *Atta sexdens* (L.) in Rio Grande do Sul, Brazil (Diehl-Flieg et al. 1992). Also in Brazil, *Metarhizium anisopliae* (Metschnikoff) Sorokin mycoses have been observed on *Atta sexdens rubropilosa* Forel and *Atta bisphaerica* Forel (Jaccoud et al. 1999). *Metarhizium anisopliae* was frequently isolated from nest soil of Panamanian *Atta* and *Acromyrmex* spp.; however, very few live, infected individuals were detected from these same nests (Hughes et al. 2004). *Beauveria bassiana* was also isolated from 50% of soil samples (n=14) from two *Atta texana* (Buckley) mounds at the Brackenridge Field Laboratory, Austin, Texas (Sánchez-Peña, unpublished observations).

From an applied standpoint, there is great interest in the development of entomopathogenic fungi as biological control agents for leaf-cutting ants in Latin America, since the attacks of these ants on economically important plants make them primary pests (Weber 1972; Cherrett et al. 1989). In the laboratory, Jaccoud et al (1999) exposed *A. sexdens rubropilosa* workers to *M. anisopliae* in mininests and observed that the ants are capable, to a large extent, of neutralizing this pathogen in their immediate environment. Several fungi (mainly *B. bassiana* and *M. anisopliae*) have been applied in the field for control of leaf-cutting ants. High worker mortality and suppression of colony foraging have been reported in some trials (Silva and Diehl-Flieg 1988; López and Orduz 2003). Leaf-cutting ants have an arsenal of defense mechanisms against pathogenic nicroorganisms. These defenses include recognition and behavioral reactions (grooming, cleaning, and physical transport of infectious material) (Kermarrec et al. 1986). They will attempt to remove any contaminated material from the nest, including dead or dying nest-

mates, in order to prevent spread of infection to other members of the colony (Kermarrec et al. 1986). Ants in general will engage in intensive self-grooming to rid themselves of any alien fungal spores (Kermarrec et al. 1986; Diehl-Fleig and Lucchese 1991; Sánchez-Peña and Thorvilson 1992). Spores of fungi are accumulated into small pellets in the infra-buccal pocket (Kermarrec et al. 1986), where they are exposed to chitinolytic secretions from the labial glands, which inhibit their germination (Febvay et al. 1984). In addition, both the ants and their mutualistic fungus are reported to produce various antibiotic compounds (Jaffe et al. 1994; Kermarrec et al.1986, Poulsen et al. 2002a, 2002b) that help prevent the survival of antagonistic and entomopathogenic fungi (*Metarhizium*) and other microorganisms in the nest environment.

In this chapter, I investigated the susceptibility to infectious disease of the Mexican leaf-cutting ant, *Atta mexicana* workers raised to adulthood on colonies cultivating either an *A. mexicana* or a *T. zeteki* fungal symbiont. The experimental infection tested was mycosis caused by *Beauveria bassiana* (Hyphomycetes: Moniliales). In this way, trophic systems were experimentally analyzed at the fourth trophic level. The two trophic systems (differing on having either an *Atta* or a *Trachymyrmex* symbiotic fungus) were as follows: plant--fungal cultivar--ant--entomopathogen; and they were analyzed at the ant-entomopathogen level of interaction. In this chapter, it was expected that ants reared on a non-native, presumably less derived symbiotic fungus would be negatively affected in their defense against pathogenic fungi, rendering them more susceptible than workers reared on the native fungal symbiont. The observations reported on Chapters 3 and 4 indicate that cultivation of this novel symbiotic fungus has severe, negative nutritional consequencies for the ants (e.g., smaller worker size).

Environmental (including nutritional) stress is often associated with reductions in immunocompetence (Schmid-Heimpel 1998). Herein, immunocompetence is used to refer to "a general capability to resist pathogens and parasites" (Schmid-Heimpel 1998). In general, nutritional stress increases the susceptibility of insects to infections. Food-deprived workers of the leaf-cutter ant *Acromyrmex octospinosus* cease to produce antibiotic secretions from their metapleural glands (Bot and Boomsma 1996; Poulsen et

al. 2002b). These results were indicative of the cost of this immune mechanism on the ants'physiological economy. Donegan and Lighthart (1989) found that starvation and nutrition stresses increase the susceptibility of *Chrysoperla carnea* (Neuroptera: Chrysopidae) to *B. bassiana*. Appropiate nutritional levels increase the resistance of the leaf-cutter bee, *Megachile rotundata* (Hymenoptera: Megachilidae) to infection by the chalkbrood fungus, *Ascosphaera apis* (Ascomycota: Ascosphaereales) (Goettel et al. 1993); similar effects were observed in honeybees, *Apis mellifera* (Hymenoptera: Apidae) against American foulbrood, *Paenibacillus larvae* (Bacillales: Bacillaceae)**;** pollen feeding reduced the mortality rate of workers (Bamrick 1964; Rose and Briggs 1969; Davidson 1973). In other, non-social insect systems, favorable nutritional status also generally increases resistance to infections (Schmid-Hempel 1998). In the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae), elevated nitrogen content in the diet reduces the incidence of infections by *Nosema fumiferanae* (Microsporida: Nosematidae) (Bauer and Nordin 1988). However, Milner and Soper (1981) reported that spotted alfalfa aphids, *Terioaphis trifolii* (Homoptera: Aphidae) subject to nutritional stress are more resistant to infection by the fungus *Zoophthora radicans* (Zygomycetes: Entomophthorales).

5.2 MATERIALS AND METHODS

5.2.1 Experimental Insects and Fungi.

The bioassay utilized workers from functional, 18- to 30-months old, queen-right *A. mexicana* colonies cultivating either an *A. mexicana* or a *T. zeteki* symbiont. These colonies were established in the laboratory as described in Chapter 3. Workers from these colonies were used for exposure to conidia of the mitosporic fungal pathogen *B. bassiana*.

5.2.2 Fungal Pathogen.

The entomopathogenic mitosporic fungus *B. bassiana* was obtained from an *A. mexicana* colony in Guadalupe, Nuevo León, Mexico, on 10 July 2002. Ant carcasses overgrown

with *B. bassiana* mycelium and conidia were collected from the surface of the external fungal dump of the colony. In this species, workers dispose of the exhausted fungal substrate and dead workers on an exposed pile adjacent to the nest mound (Sánchez-Peña et al. 2003). In this area of Mexico, these accumulations of fungal substrate and dead workers are frequently abundant on *Beauveria*-infested carcasses (Sánchez-Peña, unpublished observations).

5.2.3 Propagation of Fungus and Preparation of Inoculum for Bioassay.

The virulence of *B. bassiana* towards selected insects appears to be stable after a moderate number of successive transfers on artificial media (Sánchez-Peña and Thorvilson 1993). However, to minimize the risk of reductions in pathogen virulence from selection for saprophytism during *in vitro* cultivation, *Beauveria* was propagated *in vivo* on *A. mexicana* workers. Ant workers were topically contaminated with conidia from the original collected carcass described above. These workers were confined in plastic containers with high humidity (by placing a water-saturated piece of sponge in each container) at room temperature. Under these conditions, challenged ants usually died within 3 to 4 days and sporulation of the fungal pathogen on ants was copious seven days after exposure (similar to Figure 2.2A). If not used immediately, these cadavers were stored in the refrigerator (4-6° C). The fungus was successively passaged on ants four times after its collection in the field. For the bioassay, one-month-old fungal spores (conidia) produced in the 4^{th} inoculation were utilized. Carcasses with sporulating *B. bassiana* on them were dried for three days at room temperature and < 50% relative humidity to facilitate dislodging of spores from spore clumps and from the infected carcasses. A total of 30 ant carcasses with conidia were mixed with 3 grams of sieved, sterile vermiculite (2 mm sieve), vortexed at maximum speed for one minute, and then vigorously shaken for 5 minutes, until no clumps of mycelia or spores were visible in the vermiculite. To determine spore numbers in this granular preparation (fungus in vermiculite), 0.1 gram of preparation was shaken in 10 ml of 0.1 % Tween 20 in water and spores were counted in a improved Neubauer cell counter chamber, using standard cell suspension count methods (Caprette 2004). This stock preparation contained 1.25 x

10^8 spores/gr. This stock was serially diluted with plain sterile vermiculite to obtain a series of seven spore concentrations, as follows (spores/gr): 1.25×10^8 (stock), 0.6×10^8, 1.25×10^7, 1.25×10^5, 1.25×10^4, 1.25×10^3, and 1.25×10^2.

5.2.4 Exposure of Ants to Pathogen.

Herein, the different treatments are the different fungal symbionts that the ants were reared upon (n=2), combined with the spore concentrations of the pathogen that these ants were exposed to (n=7) (total of 14 treatments: Table 5.1). Groups of 15-25 ants were added to 50-ml plastic test tubes containing 5 ml of the vermiculite inoculate treatment. There were four replicates/treatment combination. Tubes with ants were gently turned upside down 10 times to ensure the contact of spores with the ant's cuticle. Control groups of ants were treated likewise in vermiculite containing no spores. Ants and vermiculite were then transferred quickly to plastic containers (250 ml). Water was added to the vermiculite to insure high humidity levels conductive to fungal infection. Enough water was added (2.5-3.0 ml) to saturate vermiculite in the containers, without accumulation of excess free water. Containers with exposed ants were incubated at room temperature. Ant mortality was registered 48 hours after exposure to the treatments and every 24 hours thereafter for a total of 8 days.

5.2.5 Data Analysis.

After gathering the survival data on days vs. spore concentrations for each of the two symbiotic fungus treatments, percent mortality data for each spore concentration-per-fungus combination were analyzed with the Probit regression procedure of SPSS (2003) for the probabilistic calculation of median lethal time and its confidence intervals (Sánchez-Peña and Thorvilson 1995; Todorova et al. 2002). The median lethal time (or lethal time 50, LT_{50}) is the probabilistic time lapse after which 50% of the total population will die after exposure to a specific treatment.

The slopes and intercepts of the survival lines (probit values vs. ln-transformed spore concentrations) were compared by linear regression, and by Analysis of Covariance

(ANCOVA) using the software "JavaScript E-Labs Learning Objects" of the University of Baltimore: (http://home.ubalt.edu/ntsbarsh/Business-stat/opre504.htm - rcomputeodel) and (http://home.ubalt.edu/ntsbarsh/Business-stat/otherapplets/ANOCOV.htm) (Arsham 2004), considering the symbiotic fungus treatments as main factors.

5.3 RESULTS AND DISCUSSION

5.3.1 Susceptibility as Indicated by LT_{50} Values.

In these tests, control *A. mexicana* workers were short-lived in general; their survivorship (LT_{50}) (5.43 and 4.46 days, respectively, for ants reared on *Atta* and *Trachymyrmex*-fungus) are similar to the 5 days (LT_{50}) reported by Bacci et al. (2004) for *A. sexdens* workers deprived of their symbiotic fungus and given water only (5 days). Ants were not provided with symbiotic fungus [which could have increased longevity of the controls; (Bacci et al 2004)] because leaf-cutter fungal symbionts are reported to possess antifungal propierties; thus, these symbiotic fungi could potentially interact directly with the fungal pathogen (Kermarrec et al. 1986). Mortality rates were significant and followed the expected inverse relationship between spore concentration and survival time (P=0.0153 and 0.0296, respectively, for the *Atta* and *Trachymyrmex* fungal symbiont treatments) (Tables 5.1 and 5.2; Figure 5.1A and 5.1B). *Beauveria* sporulated heavily on the dead challenged ants; it was reisolated from these carcasses, and it sporulated in at least 50% of them, confirming its development and assumed lethal action.

The ANCOVA did not detect significant differences between the slopes and intercepts of the spore concentration/survival responses of the two symbiotic fungus treatments (Table 5.2). This indicates that the survivals of the ants upon pathogen challenge were not significantly different and, more importantly, independent of the specific symbiotic fungus (*Trachymyrmex* or *Atta*) that they were reared upon as larvae and adults. The Probit analysis supported this conclusion. Several of the LT_{50} values and their confidence intervals overlapped or nearly did so. In no case did the same conidial treatment cause faster mortality (i.e. resulted in a smaller LT_{50} value) for the workers reared on the *Trachymyrmex* fungus than for those reared on the *Atta* fungus. (Table 5.2 and Figure

5.1). Ant larval development by feeding on a novel and possibly less derived, fungal symbiont than their own did not seem to cause a higher susceptibility nor an apparent weakness of the adult ants to the pathogen *B. bassiana* compared to ants that developed feeding on their native *A. mexicana* fungal symbiont. Therefore, if there was an effect of the different fungal symbionts used as colony food, it was in the opposite direction than expected, since in s→everal treatments (spore concentrations) *Trachymyrmex* fungus-reared *Atta* ants lived *longer* than *Atta* fungus-reared *Atta* ants. This was somewhat surprising considering the radical differences in worker size and number between native and fungus-switched colonies. I expected that at least some of the physiological mechanisms involved in the defense against pathogenic fungi (see below) would be negatively affected in the workers from the switched colonies.

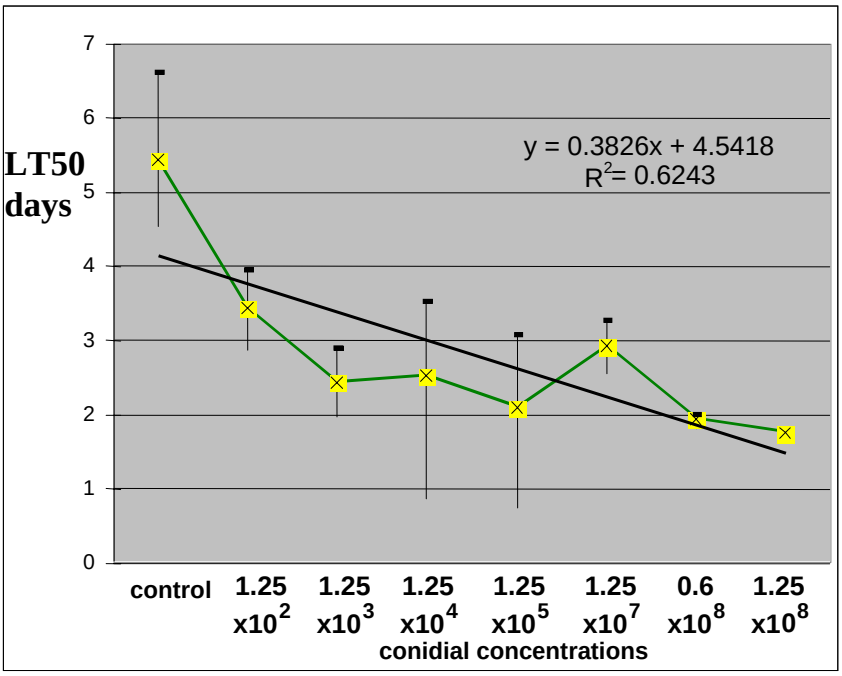
The fungus-switched ant colonies appeared to be facing significant chronic nutritional stress (Chapters 3 and 4). There are numerous immunological mechanisms in insects that could be weakened by this kind of pressure. Insect immune systems are highly developed and successful pathogens of insects must be able to circumvent and overcome these systems. Mechanisms against microbes in insects include cellular immune responses, divided into multicellular nodule formation and encapsulation involving both granular cells and plasmatocytes, and phagocytosis by individual hemocytes (Hung et al. 1993; Miller et al. 1994; Lord et al. 2003). There are also humoral immune responses (those mediated directly by molecules in the hemolymph, like melanin). Regarding entomopathogenic fungi, there are compounds (like fatty amides) on the surface of the cuticle of insects that can affect the adherence, germination, and growth of spores (Lord et al. 2003). For a review of the different immune responses of insects to entomopathogenic fungi, see Gillespie et al. (2000). The fungus *B. bassiana* is capable of modulating the immune response of its insect hosts in a highly specific way; for example by mechanisms that enable the fungus to go undetected by the insect's phagocytes in the hemolymph. During this pathogen's attack, it is frequent that the insect host recognition and signaling mechanisms are evaded; an example of evasion is the production of wall-less hyphae that escape recognition by the insect's immune system (Hung et al. 1993).

Moreover, even if *B. bassiana* is detected, insect cellular defenses (nodule formation or encapsulation) are insufficient to suppress fungus development if the fungus' propagules are present above a numerical threshold. This pathogen is also able to overcome non-cellular (humoral) encapsulation (i. e. deposition of extracellular material around invading bodies) (Gillespie et al. 2000).

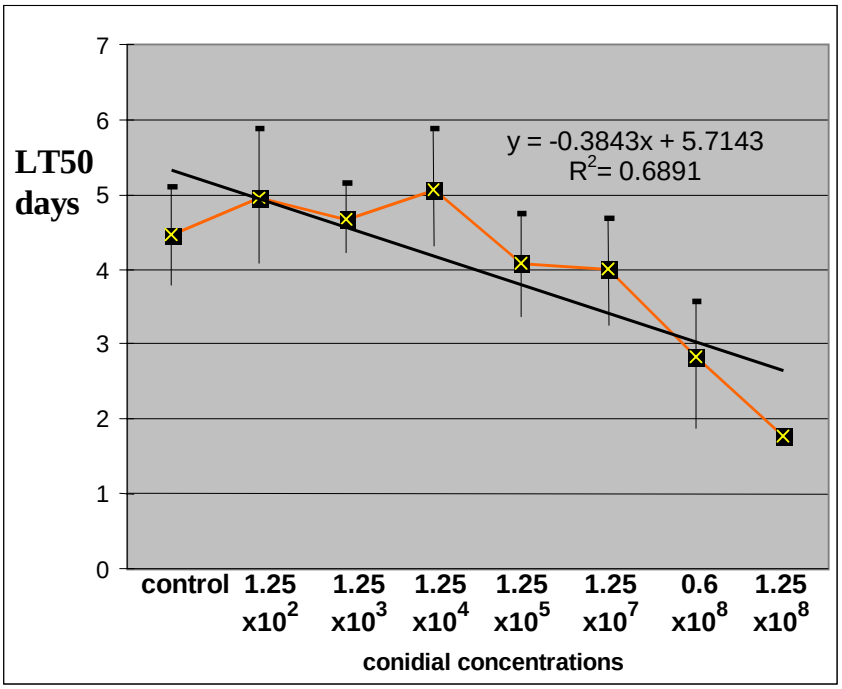
This experimental challenge with *B. bassiana* indicated that workers raised on colonies cultivating the "primitive" symbiont (i.e. switched colonies) were as susceptible as workers from colonies harboring the native fungal symbiont. Switched colonies appeared to be under considerable chronic (likely nutritional) stress (Chapters 3 and 4); however, these colonies appeared to adapt themselves rather well to this apparent nutrient limitation, at least regarding resistance to *B. bassiana* fungal disease. Indeed, switched ants sometimes seemed to be more resistant than non-switched ones, although these differences were not significant (Table 5.1; Figure 5.1). These results suggest that the "stunting" induced by the less derived fungal symbiont (Chapter 4) might not be due to simple starvation, since starved ants might be expected to be more susceptible to disease (Schmidt-Hempel 1998). An explanation is that the novel (*Trachymyrmex*) symbiotic fungus induced altered, but relatively benign, regulation of development in the ants facing nutritional stress. Thus, larval feeding on the novel fungal symbiont perhaps triggered the production of smaller but otherwise "normal" individual ants.

Another, perhaps non-exclusive explanation of these particular trophic relations (effect of nutrition on pathogen action) is that the ant's homeostasis at the colony level (behavioral or physiological homeostasis) is responsible for optimizing utilization of nutrients when these are limiting, producing fewer, smaller, but healthy workers. Still another possibility is that nutritional stress in these ants renders them less susceptible to fungal invasion, as reported in the alfalfa aphid-entomopathogenic fungus system (Milner and Soper 1981). The causes of this reduced susceptibility are unknown; maybe synthesis of antibiotic substances is increased under stress.

Figure 5.1A and B. Lethal Time 50 (LT_{50}) values for *Atta mexicana* workers.



5.1A. Mortality of workers reared on *Trachymyrmex zeteki* fungus.



5.1B. Mortality of workers reared on *Atta mexicana* fungus.

Figure 5.1 continued. *Atta mexicana* workers were exposed to different concentrations of the entomopathogenic mitosporic fungus *Beauveria bassiana*. Conidial concentrations are conidia/gr of vermiculite. Bars indicate LT_{50} values and upper and lower confidence intervals. A, Workers reared in colonies cultivating an *Atta mexicana* fungal symbiont; B., Workers reared in colonies cultivating a *Trachymyrmex zeteki* fungal symbiont.

Table 5.1. Linear R^2 and Analysis of Covariance (ANCOVA) slope and intercept values for survivorship of *Atta mexicana* workers after exposure to different concentrations of *Beauveria bassiana* conidia.

	R^2	**Slope**	**std. error of slope**	**Intercept**	**std. error of intercept**	**P**
ants reared on *Atta* fungus	0.6243	-0.1437	+/- 0.042	4.3667	+/-0.5312	0.0153
ants reared on *Trachymyrmex* fungus	0.6891	-0.1287	+/- 0.045	5.3693	+/-0.5636	0.0296

Ants were reared on either an *Atta mexicana* (native) or a *Trachymyrmex zeteki* (novel) symbiotic fungus. R^2 value is from linear regression; other values are from ANCOVA.

Table 5.2. Probit analysis: LT_{50} values (and confidence intervals) for the topical application of conidia of *Beauveria bassiana* to *Atta mexicana* workers reared to adulthood on a fungal symbiont of either *Trachymyrmex zeteki* or *Atta mexicana*.

	Conidial concentrations applied to workers (conidia/gr. vermiculite)							
	Control	1.25 $x10^2$	1.25 $x10^3$	1.25 $x10^4$	1.25 $x10^5$	1.25 $x10^7$	0.6 $x10^8$	1.25 $x10^8$
LT_{50} values for ants reared on *Atta* fungus	5.43* (4.25-6.63)	3.44 (2.87-3.96)	2.44 (1.96-2.91)	2.53 (0.8-3.52)	2.09 (0.744 -3.1)	2.92* (2.55-3.28)	1.95* (1.82-2.02)	1.76 +
LT_{50}values for ants reared on *Trachymymex* fungus	4.46* (3.79-5.11)	4.95 (4.09-5.89)	4.68 (4.21-5.16)	5.07 (4.31-5.88)	4.08 (3.36-4.74)	4.02* (3.26-4.7)	2.84* (1.88-3.57)	1.78 +

Within a column, LT_{50} values followed by an asterisk (*) are not significantly different (they have overlapping confidence intervals). LT_{50} analysis did not yield confidence intervals for the values followed by a plus sign (+).

Chapter 6: A New View on the Origin of the Attine Ant-Fungus Mutualism: The Exploitation of a Pre-existing Insect-Fungus Symbiosis (Hymenoptera: Formicidae).

Synopsis: Current hypotheses on the origin of the fungus-attine ant symbiosis propose, as an ancestral first step in the evolution of fungal cultivation, fortuitous feeding on fungi growing adventitiously on substrates like rotting wood, insect parts, seeds stored by ants in nests, regurgitated infrabuccal pellets, free-living soil fungi, or mycorrhizae. However, fungi with feeding-deterrent properties regularly colonize these substrates. *De novo* feeding on these fungi by the attine ancestor is unlikely since the almost universal presence of mycotoxins on adventitious fungi is a formidable barrier to mycophagy. In addition, there is no evolutionary history of mycophagy in the Hymenoptera, although it has been proposed that some ant foundresses might obtain nutrients from soil fungi.

Instead, I propose that attine mycophagy began from opportunistic, selective feeding on wood-colonizing fungi previously "domesticated" by other insects such as ambrosia beetles: (Coleoptera: Curculionidae: Scolytinae or Platypodinae) or less likely, woodwasps (Hymenoptera: Siricoidea). Attine ancestors foraged for beetle brood and fungal biomass in the galleries of those insects. This provided the attine ancestor with fungi that were nutritious and non-toxic in general. The invading ants' debris (exuviae, meconia, saliva, and particularly territorial fecal droplets) possibly allowed the growth of the "good" domesticated fungi in galleries, while ants mechanically eliminated undesirable fungi.

Feeding on beetle fungi first allowed the development of broad mycophagy in ants, and later of the gardening habits. Subsequently, more restricted, specific mycophagy evolved. Only after serious barriers were overcome did incipient mycophagy develop, followed by cultivation. The ambrosia fungi-lined beetle galleries in wood provide one location favorable for this sequence.

An analogous progression in mycophagy and incipient fungus manipulation, departing from the omnivore diet has occurred in *Megalomyrmex* ants, which feed parasitically on brood and fungus gardens of attine ants.

I hypothesize that colonies of the ancestral attine nested in wood and adopted mated foundress queens after mating flights, and colonies reproduced by budding. I contend that queen adoption by established colonies allowed the chronological continuation of the incipient symbiosis. This allowed to bypass the need for transportation of the proto-symbiotic fungus between mother and daughter colonies; instead, adoption of new queens allowed the continuous existence of the symbiosis in the same colony. Concurrently, sustainable explotation of the fungus developed, instead of its immediate consumption, homologous to the sustained explotation of homoptera by ants and of slave pupae by slave-making ants.

Mycophagy preadapted the ants to sample free-living fungi in wood and soil as cultivars. The fortuitous finding of more adequate lepiotaceous strains in soil allowed the adaptive radiation exhibited in present-day attines. The cultivation, -by the primitive attine *Apterostigma*-, of wood-colonizing Basidiomycota is possibly of ancestral significance.

6.1 INTRODUCTION; HYPOTHESES ON THE ORIGIN OF THE FUNGUS-GROWING SYMBIOSIS OF ATTINES

Fungus-growing ants (Hymenoptera: Formicidae: Attini) are a fascinating and diverse group comprising about 210 species in 13 genera. A monophyletic clade (Currie et al. 2003), all the Attini share the habit of cultivating mycelia of fungi [Basidiomycota: Agaricales: Agaricaceae (formerly Lepiotaceae) and Pterulaceae] for food (Weber 1972, Chapela et al. 1994, Mueller et al. 1998). Both Lepiotaceae and “lepiotaceous” will be used here to refer to the attine symbionts that were classified in that family (Lepiotaceae), which has recently been transferred to the Agaricaceae (Vellinga et al 2003). Survival of attine ants depends on the successful cultivation and production of their symbiont’s fungal biomass, which is very probably the only food of attine larvae. Fungus-growing

ants live only in the American continents. They are vastly more diverse in the warm, humid rainforests of Central and South America (Weber 1972, Garcia et al 2002).

Although fungus-growing ants in general are usually conservative cultivators, they may change their original fungal symbiont stocks occasionally when catastrophic events lead to the loss of their original cultivar (Chapela et al. 1994, Mueller et al. 1998, Adams et al. 2000).

The main hypotheses on the origin of the fungus-attine ant symbiosis were thoroughly reviewed by Mueller et al. (2001). These conjectures propose, as a first step towards cultivation, fortuitous ancestral feeding on adventitious fungi growing on nest walls, rotting wood, insect parts, insect feces, seeds stored by ants in their nests, regurgitated infrabuccal pellets, free-living fungi in soil, or mycorrhizae. All these proposals seem to imply more or less selective feeding upon the fungi growing on these substrates. However, almost all of the substrates are regularly colonized by a very diverse assemblage of fungal species, many of which are often toxigenic or have feeding-deterrent properties (Domsch et al 1980, Wicklow et al. 1994, Joshi et al. 1999) and/or are ecologically obligate plant symbionts (mycorrhizae). The toxic nature of most adventitious fungi has been recognized for a long time: Majumder et al. (1964) listed no less than 64 complex toxic metabolites from species of *Aspergillus* and 97 from *Penicillium*, and lesser numbers from the fungal genera *Absidia, Alternaria, Cephalosporium, Curvularia, Epiccocum, Fusarium, Giberella, Mucor, Phoma, Rhizopus, Stemphylium* and *Trichoderma.*

All ant species form tiny pellets (infrabuccal pellets) and store them temporarily in a specialized buccal pocket. Ants carry these pellets around in their mouths. Eventually they discard the pellets; other ants accumulate food particles in these pellets and feed their larvae with them. Bailey (1920) first proposed and Mueller et al. (2001) expanded the concept that ants started feeding on fungi growing on these infrabuccal pellets. In their hypothesis, fungal cultivation by attines developed from the buccal transporting and dispersal of fungi by the ants. They also suggested the possible existence of a hitherto

unknown group of mouth-transported and dispersed Basidiomycota that could exist associated with ants.

Fungal growth does form on these pellets (Quinlan and Cherrett 1978). However, this collecting of particles (including spores) in the infrabuccal pellet is primarily a cleaning mechanism, ants being meticulously self-grooming insects (Schultz and McGlynn 2000). These pellets are usually formed after ants lick surfaces, and, as a result, they show a wide array of fungal propagules so collected. The microbes growing from these pellets are a diverse lot of contaminant types (Quinlan and Cherrett 1978). In the ant *Acromyrmex octospinosus* (Reich), the pellet microflora is characterized by a great abundance and diversity of microbial species, including bacteria and even nematodes; it also lacked constant, dominant species (Quinlan and Cherrett 1978, Febvay and Kermarrec 1981). Ant infrabuccal pellets also include the ubiquitous soil fungus *Mortierella* (Mueller et al. 2001); ingestion of *Mortierella wolfii* Mehrotra and Baijal by cattle can cause abortions (Ribes et al 2000). Detailed micrographs of contents of pellets (Bailey 1920) show abundant hyaline and dematiaceous (dark-pigmented) hyphae. Moniliaceous (light-pigmented or non-pigmented) fungi, like *Penicillium* and most *Aspergillus* spp. are difficult to detect in these images. However, the images clearly show abundant conidia (mainly dematiaceous) like those of *Cladosporium, Alternaria, Fusarium, Stemphylium, Cercospora* and *Helmintosporium sensu lato*. All of them are widespread, fast growing, aggressive substrate colonizers, and powerfully toxigenic fungi (Majumder et al. 1964, Domsch et al. 1980, Wright et al. 1982, Scott et al. 1985, Dowd et al. 1988, Munkwold and Desjardins 1997). I maintain that feeding on diverse assemblages of adventitious fungi is a formidable challenge for any potential novel mycophagist. In fact, there are very few if any mycophagous insects feeding on these ubiquitous molds. Possible exceptions are some Coleoptera like the Latriididae and some Endomychidae (Lawrence 1989, 1991, Blackwell 2002) but the specific biology, food habits and actual fungal host species of these beetles are very poorly known.

It can also be argued that the theoretical myrmecochorous, “buccophilous” fungal propagules within these pellets would need to have had physical or chemical adaptations

to avoid complete digestion by ants, as ant-transported seeds do (Gómez and Espadaler 1998, Rodgerson 1998). Ant-transported seeds have hard seed coats that prevent embryo consumption but reward transporting ants with edible, disposable external appendages such as elaiosomes (Gómez and Espadaler 1997, Rodgerson 1998). The hypothetical ant-dispersed fungal propagules (spores?) would most likely lack tissue and organ differentiation and would probably be unicellular, unlike plants. Thus, protection mechanisms (feeding deterrents, etc.) to prevent their complete consumption by ants would have to exist at the individual cell level, posing a conflict between dispersal and ant reward (ingestion) for these hypothetical myrmecochorous fungi. More importantly, these diffuse feeding relationships between different fungal species and ants are more likely to have resulted in ant taxa specialized as fungal grazers and not necessarily as fungal cultivators. With few possible exceptions (e.g., a *Camponotus* sp., Schultz and McGlynn 2000; Mueller et al. 2001), essentially no ants feeding on free-living fungi have been reported, although it has been speculated that some ant foundresses could obtain nutrients from fungal hyphae in soil (Malyshev 1968 in Mueller et al. 2001). Ants are universally repelled by adventitious molds and generally avoid all close associations with fungi; they have evolved mechanisms that discourage the presence of fungi in their nests (Kermarrec et al. 1986; Schultz and McGlynn 2000). From the information available, ants universally reject fungi as food and as inquilines in their living quarters, although this generalization merits further investigation.

6.2 INSECT MYCOPHAGY AND FUNGAL METABOLITES IN DEFENSE AGAINST PREDATORS

The fungi colonizing buccal ant pellets belong to groups with highly developed chemical defense mechanisms against arthropod predation. Mycotoxins are fungal secondary metabolites that are either acutely toxic to animals or show other adverse manifestations (i.e., mutagenicity, teratogenicity, or carcinogenicity) (Wicklow 1988). Fungal toxins can prevent or reduce loss of fungal resources by animal feeding; fungal toxins are considered analogous to chemical defenses of higher plants in deterring potential predators (Janzen 1977). Predation as a selective force has shaped the chemical defense

systems of fungi (Wicklow 1988). Fungi and insects commonly compete for resources in both natural and agricultural situations (Cotty 1991) and their respective developments are frequently incompatible. Insects are susceptible to many fungal toxins they find in real-life situations (i.e., Dowd et al. 1988). To avoid being eaten by insects and other animals represents an obvious advantage for competing fungi. Detritivorous insects are better able to tolerate or utilize moldy and mycotoxin-contaminated resources than are herbivorous insects (Wicklow 1988). Whittaker and Feeny (1971) suggested that toxic chemicals produced by fungi might afford relative protection against potential predators such as nematodes and arthropods that consume soil and litter fungi, or vertebrate mycophagists that consume mushrooms. Interestingly, cyclic peptides, a type of fungal toxins, are found both in many unrelated Basidiomycota (Agaricales) (*Amanita, Galerina, Lepiota)* and in insect-pathogenic Hyphomycetes; in the latter group, they play a role in pathogenesis upon the insect hosts (Dowd 1999).

For dispersal, some toxigenic fungi are loosely (not strictly) associated with insect vectors. Thus, there is a potential conflict in such facultative insect-fungal associations. Janzen (1977) proposed that the ideal strategy for a fungus would be to produce a small toxic, dangerous zone around itself, yet not to exclude larger insects that might aid in the fungus dispersal. On the other hand, many or even most of the ubiquitous fungi passively disperse their spores on air currents, water, soil, et cetera.

6.3 WHY ARE THERE SO FEW MYCOPHAGOUS ANTS?

The paucity of truly mycophagous ants other than attines and *Megalomyrmex* (see below) is intriguing: Fungal biomass (hyphae, stromata, sclerotia, rhizomorphs in litter, ephemeral and perennial fruiting bodies, lichens, spores, etc.) is both abundant and widespread (although the abundance of large fungal fruiting bodies is highly variable in space and time) where ants are most abundant and diverse in both tropical and warm temperate humid ecosystems. Fungal biomass in the environment is probably ubiquitous enough, and, apparently, rewarding enough when found to provide a pressure on ants to use it – a colony will almost certainly be presented with the opportunity to eat fungus at

some point. However, very few, if any, ant species exploit this resource (Wheeler 1910, Hölldobler and Wilson 1990, Schultz and McGlynn 2000, Mueller et al. 2001). On the other hand, because fungi are rich in animal- and insect-toxic compounds (Wicklow and Cole 1982, Wicklow 1988, Dillon et al. 1992, McNicholas et al. 1996, Belofsky et al. 1998a, 1998b, Simpson 1995, Churchill et al. 1998, Oh 1998a, 1998b, Joshi et al. 1999), and because land-locked, wingless insects have comparatively limited search and dispersal capabilities, there is probably good reason for the lack of fungivorous ants. The almost universal non-utilization of fungi as food by ants probably indicates significant deterrence, defenses, and/or lack of nutritional value in fungi, which prevent indiscriminate feeding by ants. The chemical versatility of fungi probably precludes indiscriminate *de novo* feeding for most animals, especially on diverse assemblages of common fungi such as those found in the infrabuccal pellets of ants (Bailey 1920, Quinlan and Cherrett 1978, Mueller et al. 2001 and references therein). Because ant workers are never winged, ant populations are limited in their capability to exploit an abundant but highly dispersed and often sporadic resource such as fungal biomass. Flying insects such as beetles and flies do use fungal biomass, possibly because they disperse effectively to find scattered sources of food. Non-flying arthropods also use fungi as food, but they are minute organisms (Collembola, Thysanura, and soil mites) that belong to very ancient (Paleozoic), ancestrally saprophagous, soil-dwelling lineages (Hopkin 1997, O'Connell and Bolger 1997). They can sustain their whole lifecycle (perhaps for several generations) even in small, localized amounts of fungal biomass such as hyphae in soils (Hopkin 1997, O'Connell and Bolger 1997, Hubert et al. 2000). Besides, their small size, sensory organs and feeding mouthparts allow them to probe and feed down to individual hyphae. Thus, they are well adapted to sort among individual hyphae in soil or other habitats.

6.4 INSECT MYCOPHAGY IN THE HOLOMETABOLOUS ORDERS: ANCESTRAL FEEDING HABITS AND POSSIBLE IMPLICATIONS FOR FUNGAL CONSUMPTION IN THE DIPTERA, COLEOPTERA AND HYMENOPTERA

It is possible that the ability to produce mycotoxins in fungi is mainly a response to deter potential arthropod predators, particularly in warmer latitudes. Wicklow (1990) and Wicklow et al. (1994) proposed that pressure to avoid insect feeding has been the main factor selecting for toxin synthesis in widespread species of *Aspergillus*. They suggested that insects and not plants are the key to the regulation of aflatoxin biosynthesis in these fungi. Nearly all fungal compounds classified as cytotoxic or neurotoxins are also toxic for insects (Wright et al. 1982). In several fungal species toxigenic strains are more common in tropical latitudes (Wicklow and Cole 1982), where insects are most abundant. There are no known examples in which toxic fungal metabolites have been shown to be nutritionally beneficial to invertebrate mycophagists (Wicklow 1988) as happens in many plant-insect interactions.

Unlike for the Diptera and Coleoptera, two holometabolous insect orders with many mycophagous members, there is no evolutionary history of saprophagous habits or mycophagy in the Hymenoptera. The whole order is primitively phytophagous with a single switch to insectivory in the suborder Apocrita, which includes the ants (Formicidae) (Gauld and Bolton 1988, Sudd and Franks 1987, Vilhelmsen 1997, Whitfield 1998). The bees (Apidae) are a recent apocritan group (arising 30-40 mya) that reverted to phytophagy and have abandoned the ancestral carnivorous behavior of the sphecoid wasp clade (Gauld and Bolton 1988, Michener 2000).

The Coleoptera includes many mycophagous species, but, unlike the Hymenoptera, it is very probable that saprophagous habits on decaying organic material are ancestral among beetles (Crowson 1981, Lawrence 1989, Betz et al. 2003). The basal beetle suborder Archostemmata has fossils in the Paleozoic (Permian); it includes the very primitive, extant families Ommatidae, Crowsoniellidae, Micromalthidae and Cupedidae. These are

all saprophagous, feeding on decaying wood, sometimes in soil (CSIRO 1997, Farrell 1998, Caterino et al. 2002). The primitive Diptera share such nutritional habits. The basal dipteran suborder Nematocera includes many saprophagous and mycophagous species (McAlpine et al. 1981). Some dipterans are very efficient fungus-feeders, being able to feed on *Amanita* mushrooms that are extremely toxic or deadly to most other organisms (Jaenike 1985).

Ancestral Hymenoptera are believed to have been phytophagous on living plants. No lineage of the Hymenoptera is saprophagous or mycophagous, aside from the Attini, some *Megalomyrmex* spp. (Formicidae) (see below) and the Siricidae (Whitfield 1998, Gauld and Bolton 1988). It is possible that some of the enzymes used to detoxify plant secondary chemicals (as would be produced by phytophagous insects like ancestral hymenopterans) could be useful in digesting fungi. Some mammalian herbivores (ruminants, like deer, reindeer and caribou) feed on mushrooms and lichens, but possibly aided by their gastric and intestinal microflora (Klein 1982). However, to my knowledge there are no reports of insect species feeding on both healthy, sound tissues of vascular plants, as well as on fungi.

6.5 BROAD AND RESTRICTED MYCOPHAGY; SCENARIOS FOR *DE NOVO* CONSUMPTION OF FUNGI

Single species of mycophagous beetles and flies usually can feed simultaneously across several families or even orders of fungi; for example, the basidiomycetous fungal orders Russulales, Agaricales and Boletales (Fogel and Peck 1975; Jaenike and Selander 1979, Bunyard and Foote 1990a, 1990b). I propose that feeding on beetle-domesticated fungi induced the development of analogous broad mycophagy in ants. Broad mycophagy is herein conceived as the capability of feeding on species across the Basidiomycota and possibly even the Ascomycota. Basidiomycota and Ascomycota are sister groups and form a natural, monophyletic clade within the non-flagellate fungi (Alexopoulos et al. 1996, Berbee and Taylor 2001).

This broad mycophagy is common and prevalent in insects that utilize fungi as food. For example, *Drosophila falleni* Wheeler is a mycophagous, polyphagous species breeding in a number of mushroom genera, including highly toxigenic species of *Amanita* that are deadly for most animals (Jaenike and Selander 1979). Genetic (allozyme), ecological and rearing observations did not support the existence of host (mushroom) races, but rather that of ecologically generalist, polyphagous populations of flies developing across different mushrooms in the Northeastern USA (NY state) (Jaenike and Selander 1979). The *D. falleni* population studied bred indiscriminately on both edible (for humans), "delicious" species (*Amanita rubescens* (Pers. ex Fr.) S. F. Gray) and deadly poisonous ones (the "Destroying Angels", *Amanita bisporigera* G. F. Atk. and *Amanita virosa* (Fr.) Bertillon) (Miller 1973, Jaenike and Selander 1979, Pace 1998). Only about five species of mycophagous *Drosophila* co-occur in New York State, a number much smaller than the hundreds of species of mushrooms that are present and utilized by these flies (Jaenike and Selander 1979).

When analyzing the hypothesis of the origin of fungiculture in insects, it is important to compare the possible scenarios by which these symbioses are proposed to have developed. On one hand, the organic matter in soil includes a diverse array of ephemeral, often very toxigenic fungal and bacterial species (Bailey 1920, Janzen 1977, Domsch et al 1980, Wicklow 1990, 1994). On the other hand, newly bored galleries in sound wood are practically sterile. Except for the ecologically obligate plant parasitic endophytes that are usually toxic to insects (Bacon 1995), the microbial flora in these galleries is mostly limited to the microbes that the boring insect has brought with itself into them. If the wood being bored is deeply colonized by fungi, they are relatively restricted to specific groups. In terms of species number, they are overwhelmingly in the Basidiomycota (Fergus 1960). The potential effect of feeding on these two widely different groups of fungi (adventitious soil molds versus wood-dwelling Basidiomycota) upon novel mycophagists must be considered. Likewise, the use of previously domesticated insect-symbiotic fungi by ants, as opposed to trial- and error feeding on free-living fungi, would

have bypassed costly, risky testing, and would have provided them directly with more highly nutritious, and/or less toxic fungal species or strains.

6.6 INSECT-FUNGUS SYMBIOSES PREDATING THE CULTIVATION OF FUNGI BY ANTS

6.6.1. The Siricoidea: Chronology, General Biology.

. The Siricoidea (Hymenoptera) (horntails or wood wasps) is a fungus-symbiotic superfamily in the basal suborder Symphyta, and is more ancient than the attines (Gauld and Bolton 1998, Vilhelmsen 2000). The superfamily includes the Anaxyelidae, Siricidae and Xiphydriidae. The Siricidae and Xiphydriidae each have about 80 species of medium- to large-sized wasps worldwide. The Siricidae is made up of the subfamilies Siricinae and Tremicinae. All Siricidae and Xiphydriidae are symbiotic with fungi in a manner analogous to ambrosia beetles: larvae feed on fungal biomass in their galleries. Both families have generally similar habits. Females have long ovipositors and lay eggs deep in wood. In doing so, they also inject spores of symbiotic Basidiomycota essential for larval development (Smith and Schiff 2002). The fungi develop in the larval galleries and are consumed by the wasp larvae. There is some disagreement on the amount of fungus consumed by larvae (Smith and Schiff 2002) However, in *Xiphidria* and *Tremex* (Xiphydriidae and Siricidae: Tremicinae, respectively) the amount of mycelium eaten with the wood seems to be greater than in the Siricinae (Kajimura 2000). Regarding their fungal symbionts, in the Siricidae the subfamily Siricinae develops in gymnosperms and its species are associated with the fungus *Amylostereum*. The Tremicinae develops in angiosperms and their symbiont is *Cerrena*. The phylogenetic affinities of these symbionts are not clear (*Amylostereum* is in the Corticiales: Corticiaceae; *Cerrena* is in the Poriales: Poriaceae), but both of these fungal orders are also polyphyletic (Petersen 1995).

There is little biological information on the Xiphydriidae. The xiphydriid woodwasps develop on angiosperms only. The identity of the xiphydriid fungal associate(s) is

unknown (Kajimura 2000). Also unknown are the general biology and hosts plants of the Neotropical xiphydriids. In the Western hemisphere, the Siricidae extend south to Cuba and northern Central America. There are 4 genera and 17 species of xiphydriid woodwasps in Central and South America.

No extant siricid wasps are native to South America which makes them less likely candidates as fungal "donors" in the origin of fungus mutualism in the attines (but see Fidalgo and Smith (1987) for extinct South American siricids). On the other hand, the siricid extinction in South America could explain the disappearance of any associated host-specific, non-agricultural, mycophagous attine ancestor (analogous to *Megalomyrmex*; see below).

I will not discuss these wasps further, although their possible role as fungal donors in the attine symbiosis should be considered, since most of the interactions proposed between ants and ambrosia beetles could have also occurred between these wasps and the ancestral attines.

6.6.2 Ambrosia Beetles (Coleoptera: Curculionidae).

Besides the Attini, no other insect-fungus symbiosis is as diverse and widespread as in the wood and bark beetles in South America. The more than 7000 species of bark and ambrosia beetles (Curculionidae: Scolytinae and Platypodinae) are both abundant and diverse in the humid tropical regions of the world. The Platypodinae are especially diverse in these regions, where they comprise more than 1000 known species (Schedl 1972, Farrell et al. 2001).

These two beetle groups show marked similarities in their interactions with fungi. Each of them includes "ambrosia beetles" *sensu* Crowson (1981). They include Scolytinae and Platypodinae in which the adults construct a more or less extensive burrow system (usually in the xylem) and introduce into it specific types of symbiotic fungi ("ambrosia fungi") which form a sort of carpeting growth on the walls of the tunnels. Ambrosia beetles lay their eggs in the tunnels, and larvae feed on the fungal growth. While all

species of Platypodinae are reported to be obligately mycophagous, not all species of Scolytinae are known to be so, but few of them have been carefully examined in this respect. Some species of this beetle guild have sometimes been called "xylomycetophagous", but this concept is misleading since the contribution of wood to beetle nutrition is thought to be negligible (Browne 1961) and development depends wholly or almost wholly upon ingestion of fungal biomass. Beaver (1989) considered that the majority of tropical Scolytidae and almost all species of Platypodidae are xylomycetophagous. The Platypodinae generally breed in large-diameter, fresh host plants. Mated pairs tunnel into heartwood and introduce ectosymbiotic fungi into their tunnels upon which they and their brood feed. For the most part, the wood is not actually consumed by the beetles. The Platypodinae can only breed in undegraded, live or recently killed host material with high moisture content. Decaying wood or dried-out wood is usually unsuitable for beetle development (Crowson 1981, Wood 1982, Atkinson 2000).

As a group, the Scolytinae exploit many parts of the anatomy of vascular plants (Atkinson and Equihua-Martínez 1986a, 1986b). While in temperate forests the phloem habitat is prevalent among the Scolytinae, in tropical rain forests that seems not to be the case. Many tropical Scolytinae bore into the wood (Browne 1961, Atkinson and Equihua-Martínez 1986b). For brood development, many Scolytinae are also dependent on fungi they introduce into the galleries they bore for oviposition and their larvae feed on these symbiotic fungi. Many other Scolytinae that develop not in the wood (xylem) but in the inner bark (phloem) of trees are also mycophagous with the larvae of these beetles depending on fungi (Klepzig and Wilkens 1997, Klepzig et al. 2001a, 2001b). These beetle-fungus systems include many economically important tree pest beetles (e.g., *Dendroctonus*, *Ips*, *Scolytus*) that have been more closely examined. In these better-known systems, the presence of non-symbiotic, non-"ambrosia-like" ascomycetous fungi, like "bluestain' of wood (*Ophiostoma* spp.), is deleterious for the beetles and results in arrested or aborted larval development (Klepzig et al. 2001a, b).

The ambrosial (or symbiotic fungus-carrying) beetles are extremely diverse in the tropics. There is, e.g., an assemblage of species of Scolytinae developing in the petioles of fallen

leaves on the rainforest floor (Browne 1961). Virtually nothing is known of the biology of this and other ambrosia beetle guilds. While individuals of temperate species of ambrosia beetles are small (< 2 mm), tropical species can be longer than 10 mm (Browne 1961).

In the neotropics, the diversity of Scolytinae and Platypodinae is enormous. Equihua-Martínez and Atkinson (1986b) listed 99 species in 32 genera from a tropical dry forest on the Pacific slope of western Mexico. This fauna differed markedly from that of humid lowland forest in southeastern Mexico and Central America (Atkinson and Equihua-Martínez 1986a). As mentioned, most of our knowledge on the ambrosia beetle-fungus symbiosis comes from the study of species in the northern temperate-zone, particularly those that attack and kill economically important trees. There is little information on the biology and host associations of tropical species, and their ecological importance as a group in tropical forest ecosystems is virtually unknown (Atkinson and Equihua-Martínez 1986a, 1986b, Equihua-Martínez and Atkinson 1986).

Ambrosia beetles predate the Attini considerably, perhaps by 40 to 60 myr (Browne 1961, Schedl 1962, Hölldobler and Wilson 1990, Bright and Poinar 1994, Farrell et al. 2001). Certainly, the generic features of the Platypodinae were well established by the Upper Eocene (Bright and Poinar 1994) at which time mycophagous New World Scolytinae, like the Corthylini, were already present. The origin of the Scolytinae can be placed somewhere in the Upper Cretaceous and the Platypodinae must have had an even earlier origin (Schedl 1962, Bright and Poinar 1994). Recent phylogenetic studies based on sequencing data support a Cretaceous origin for the Scolytinae-Platypodinae clade (Farrell 1998).

6.7 POSSIBLE INTERACTION OF ANTS AND FUNGUS-SYMBIOTIC BEETLES: OPPORTUNISTIC FEEDING ON BEETLE FUNGI

Scolytid and Platypodid galleries and their edible fungi are a widely available resource in the temperate and tropical zones of the world. I propose that adaptation to fungal feeding

in the ancestral attines occurred when the carnivorous or omnivorous ancestor of attines started feeding on the brood and/or the fungi from these beetles' galleries. This would have provided the ants with readily available, nutritious biomass. Just as important, the fungal strains had already been tested and "approved" for insect mycophagy by the beetles. Thus, the beetle-fungal symbionts were already domesticated and presumably had undergone some evolutionary modifications towards non-toxicity (at least not for the beetles). On an evolutionary scale, ant explotation of this niche probably proceeded gradually, from predation upon beetle larvae developing in the galleries towards a mixed diet of fungal hyphae and beetle larvae, until exclusive mycophagy developed. Similarly, opportunistic ant feeding on recently abandoned galleries, after adult beetle emergence cannot be ruled out because these emergence holes are connected to brood chambers and could provide ants with direct access to the chambers and tunnels lined with some edible fungus (Sánchez-Peña, unpublished observations)

6.7.1 The *Megalomyrmex* Analogy and other Mycophagous Taxa.

A gradation from insectivory on other ants' brood to fungal feeding analogous to that proposed herein for the attine ancestor, is observed in species of the New World ants in the genus *Megalomyrmex*, (Hymenoptera: Formicidae: Myrmicinae). This is the only other *bona fide* fungus-feeding ant taxon besides the Attini (Wheeler 1910, 1925, Weber 1941, Hölldobler and Wilson 1990, Adams et al. 2000, Schultz and McGlynn 2000, Mueller et al 2001). Different species of *Megalomyrmex* show feeding habits analogous to the proposed intermediate stages towards mycophagy in a putative attine ancestor. While many species are omnivorous-insectivorous, some are "predators" upon attine fungal gardens (Wheeler 1910, 1925, Weber 1941, Adams et al 2000, J. Longino, personal communication). The feeding habits of *Megalomyrmex* species with this particular "parasitic-predacious" lifestyle (occupation and consumption of attine fungus gardens) range from a diet of attine brood and apparently fungal biomass, to a seemingly exclusively mycophagous habit, with the ants being able to thrive on attine fungi only (Adams et al. 2000, and references therein, J. Longino, personal communication). These

different feeding regimes can be interpreted as representing evolutionary steps towards full mycophagy.

Additionally, and intriguingly, mycophagous *Megalomyrmex* species show very basic fungus-tending behaviors. Occasionally they will "carry a particle of the substratum to another spot, insert it and pat it down with their fore feet" (Wheeler 1925). They can readjust the overall size and shape of their cached fungi (Adams et al. 2000). I contend that similar behaviors were probably relevant to the origin of cultivation habits in the attine ancestor. In the laboratory, an undescribed new species of *Megalomyrmex* survived for four months on attine fungi only (Adams et al. 2000). Further, this species consumed phylogenetically diverse symbiotic fungi from the attine genera *Cyphomyrmex*, *Trachymyrmex* and *Acromyrmex*. This suggested that a diversity of attine fungi could meet the nutritional requirements of *Megalomyrmex* sp. (Adams et al. 2000). It also reinforces my hypothesis that the ancestral attine likewise was probably able to feed on different fungi, at least to a limited extent, when it first switched from the ancestral ant diet to mycophagy. It should be mentioned that no *Megalomyrmex* ants seem to feed on free-living fungi.

In a parallel way, the mycophagous beetle groups Tachyporinae, Aleocharinae and Oxyporinae (Coleoptera: Staphylinoidea) show a similar evolution of feeding habits. Their ancestral habit was specialized predation on mycophilous organisms in fungi, followed by a switch on preference towards feeding on the mycelium of the host fungus itself (Betz et al 2003). The spatial association of insect prey and fungi leading to food habit changes is further underscored by analyses of beetle predation on Hemiptera (Leschen 2000, and references herein). The temporal and spatial association of sooty molds (Ascomycota: Dothideales and other orders) growing on homopteran honeydew on plants is considered a likely selective pressure towards these shifts. Shifts from ancestral mycophagy to predation on stenorrhynchan Hemiptera and back to fungus feeding have been suggested for the Coccinellidae, which includes mainly predaceous beetles. Several species of Coccinellidae feed also on powdery mildews (Ascomycota: Erysiphales) that are frequently associated with stenorrynchan Hemiptera like aphids (Aphidae), whiteflies

(Aleyrodidae), psyllids (Psyllidae), etc. on plant leaves and stems (Sánchez-Peña, unpublished observations). For coccinellids and other ancestrally mycophagous beetles now feeding on stenorrhynchan Hemiptera, "ancestral associations with sooty moulds that grow on honeydew may have mediated shifts from mycophagy to predation, rather than having ancestors that were predatory and attracted to a novel prey type" (Leschen 2000).

Megalomyrmex ants are not the only arthropods feeding opportunistically but specifically on fungi symbiotic with insects. Specialized fungus-feeding staphylinids (Coleoptera) colonize the galleries of fungus-symbiotic beetles (Crowson 1981). A diverse arrangement of mycophagous mites (Acarina) also inhabits these galleries (Blackwell et al. 1986, Lombardero et al. 2000, Klepzig et al 2001 a, b). A highly specialized lepidopteran, *Amydria anceps* Walshingam (Lepidoptera: Acrolophidae), is myrmecophilous, feeding exclusively on spent fungal garden accumulations of *Atta mexicana* (F. Smith) (Sánchez-Peña et al. 2003).

I propose that once the mycophagous habit was established in the attine ancestor, cultivation developed from incipient fungus-protecting and caring behaviors like those observed in *Megalomyrmex*. The processes of mycophagy development and tending of fungus enabled them to domesticate and afterwards fortuitously test different potential cultivars.

6.8 AMBROSIA BEETLES AND THEIR FUNGAL AND ARTHROPOD ASSOCIATES

The partially known basidiomycetous ambrosial associates of Scolytinae and Platypodinae in wood include "Aphyllophorales" (*Entomocorticium*, related to *Peniophora*) (Basidiomycota: Corticiales: Corticiaceae). In the most intensively studied ambrosia beetle symbiotic systems, those of *Dendroctonus* and *Ips* species, the role of *Entomocorticium* as the most important fungal symbiont is becoming increasingly clear (Bridges 1983, Lombardero et al. 2000, Klepzig et al. 2001a, 2001b). Ophiostomatalean

Ascomycota (*Ceratocystiopsis* and its associated anamorphs in *Ambrosiella* and *Raffaelea*) are also widespread as symbiotic fungi of ambrosia and bark beetles and can coexist with *Entomocorticium* in the same gallery. The presence of both symbiotic Basidiomycota and Ascomycota in these systems can be explained by the presence of another player, the ever-present beetle-associated mites. I propose that the original symbiosis included the beetles and only basidiomycetous associates. In this respect, the pine beetle, *Ips avulsus* (Eichhoff) is symbiotic with *Entomocorticium* (only?), and this mutualism is exploited by mites (*Elattoma*), which are able to feed on this fungus. In this case the mite seems to have adapted to feed on the basidiomycetous symbiont. However, analysis of the symbiosis of the southern pine beetle (*Dendroctonus frontalis* Zimmerman) indicates that the beetle-phoretic, mycophagous mites (*Tarsonemus* spp.) could have tipped the balance towards inclusion of Ascomycota in the original tree-beetle-Basidiomycota system. These mites, universally present as associates of Scolytinae and Platypodinae (Lombardero et al. 2000) are unable to feed on the ambrosial Basidiomycota, but they can feed and reproduce on the ambrosial ascomycetous fungi, as well as on the strongly beetle-antagonistic, tree-pathogenic ascomycetous "blue stain" (*Ophiostoma*) (Klepzig et al. 2001a, 2001b). These beetle-phoretic mites are the main vectors of inoculum (ascospores and conidia) of the beetle antagonist, but seemingly mite-mutualist, *Ophiostoma* between beetle galleries. The mites carry spores of these fungi in specialized pouches (sporothecae) (Lombardero et al 2000, Klepzig et al. 2001a, 2001b). On their body surface, the mites also carry other related, non- plant-pathogenic, non-ambrosial Ascomycota that colonize the galleries such as *Thaxteriola* (Blackwell et al. 1986). After hitchhiking on the body of the beetles, the mites inoculate the beetle galleries with these fungi. The beetles themselves carry both symbiotic ambrosial fungal types (Basidiomycota and Ascomycota) in specialized body cavities (mycangia).

Thus, mites were originally in direct competition with the beetles and their Basidiomycota species (Lombardero et al. 2000, Klepzig et al. 2001 a, 2001b). This conflicting situation likely resulted in strong selective pressure and differential beetle survival in galleries colonized by antagonistic Ophiostomatales species, versus those

colonized by less antagonistic strains. These less beetle-antagonistic ophiostomatoid associates of mites were the fungal ancestors of current ascomycetous ambrosia fungi in the Ophiostomatales: *Ceratocystiopsis*, *Ambrosiella* and *Raffaelea*.

My assumption is that ants fed upon the basidiomycetous symbionts of beetles, or on both Basidiomycota and Ascomycota. Even if ants became adapted to mycophagy upon ascomycetous fungi, this could have also "preadapted" them to feed on Basidiomycota, just as fleshy ascomycetous fruiting bodies (*Peziza*) share a mycophagous beetle and fly fauna with the Agaricales (Basidiomycota) (Lacy 1984). In the Basidiomycota, studying mycophagous microarthropod (Collembola and Acarina) assemblages, characteristic faunas were not detected for any species or any higher taxon of fungus (O'Conell and Bolger 1997). In general, insects and other animals feeding on myceliar, macroscopic sporocarps do not discriminate between Ascomycota and Basidiomycota, or phylogenetic lines, but mainly among traits unrelated to phylogeny (fleshy versus leathery, perennial versus ephemeral, and epigeous as opposed to hypogeous habit); the common mycophagous guilds are also more evident in sympatric hypogeal Basidio- and Ascomycota (Bunyard and Foote 1990 a, 1990b, Fogel and Peck 1975, Lacy 1984, Johnson 1996, Blackwell 2002, Vernes et al. 2001). When comparing insect use of fungal resources, the different abundance and diversity of epigeal macroscopic Ascomycota versus Basidiomycota must be considered; in warm temperate areas, fruiting bodies of Basidiomycota are overwhelmingly more diverse and abundant (Miller 1973, Guzmán 1982, Pace 1998); this is possibly valid for the neotropics also (Sánchez-Peña., unpublished observations).

6.9 *Apterostigma* AS A LIVING FOSSIL

In phylogenetic trees of the 12 or so attine genera (inferred from both morphological and molecular data), *Apterostigma* has the most basal position along with *Mycocepurus* and *Myrmicocrypta* (Wetterer et al. 1998, Schultz and Meier 1995, Schultz 2000, Currie et al. 2003). This very primitive genus cultivates a wide array of Agaricales (Basidiomycota) symbionts: some *Apterostigma* species utilize fungi in the Lepiotaceae, and some in the

Pterulaceae, a fungal family closely allied to the Tricholomataceae. As mentioned, both Lepiotaceae and "lepiotaceous" will be used here to refer to the attine symbionts that were classified in that family (Lepiotaceae), which has recently been transferred to the Agaricaceae (Vellinga et al 2003). *Apterostigma* spp. cultivate two groups of pterulaceous-tricholomataceous symbiotic fungi, referred to as G2 and G4 in Villesen et al. (2004). Other species cultivate lepiotaceous fungi related to the G3 cultivars of the lower attine genera (Chapela et al. 1994, Mueller et al. 1998, Villesen et al. 2004). *Apterostigma* spp. utilize wood chips to grow these pterulaceous symbionts (Wheeler 1910, Weber 1972, Munkacsi et al. 2004). The pterulaceous cultivars (formerly considered to be tricholomataceous: Chapela et al.1994, Mueller et al. 2001) have recently been shown to be sister taxa to the "coralloid" fungal genera *Pterula* and *Deflexula*, often reported from wood substrates. These cultivars are also related to *Gerronema, Megacollybia* and other lignicolous gilled mushrooms (Agaricales: Tricholomataceae) (Fergus 1960; Moncalvo et al. 2000; Munkacsi and McLaughlin 2001; Villesen et al. 2004; Munkacsi et al 2004). The Lepiotaceae are soil and litter fungi (Mueller et al. 2001). Regarding the identity of the ancestral *Apterostigma* cultivars, it is contended that the most basal *Apterostigma* spp. are possibly those that cultivate lepiotaceous G3 fungi; however, within *Apterostigma*, both ant species and their fungal symbionts, and their ecology, are too poorly known to assign ancestral and derived evolutionary positions to both ants and their fungal symbionts (Villesen et al. 2004). I propose that the ancestral, *Apterostigma*-like mycophagous ants eventually moved from sound and rotten wood to soil and concurrently found other, apparently more adequate lepiotaceous cultivars and adopted them. These more apt cultivars developed strong ties with their ant hosts and became almost universally prevalent as symbiont strains among the Attini, allowing the impressive adaptive radiation leading to the current diversity in attine functional lifestyles, from detritovores to polyphagous herbivores (Kaspari 2001). The progressive domestication (or selection of variants from already domesticated strains) of successively more "efficient" lepiotaceous strains allowed the origin and development of the higher attines (*Atta* and *Acromyrmex*) with their huge colonies and high nutritional requirements. However, at least a few *Apterostigma* species maintained

the ancient wood-growing cultivars as a relic. That the Pterulaceae-growing ant clade hasn't departed much, in any obvious way, from the ancestral state, while the Lepiotaceae-growing ants did, further reinforces the possibility of those symbioses being more similar to the original, ancestral attine ant and fungus.

With regard to cultivar and ant specialization, the trend across the tribe Attini is that the more derived the cultivar, the larger and more complex the ant colonies. This also holds across groups within the Attini (i.e., the higher attines: Weber 1972). Thus, there is a paradox in keeping with the current hypothesis considering the lepiotaceous cultivars as ancestral, since in general, *Apterostigma* has the least populous colonies of all attines (Weber 1972). Further, the Lepiotaceae-growing Apterostigmas (*A. auriculatum* Wheeler) have larger and more complex colonies than most of the Pterulaceae-growing ones (*A. pilosum* Mayr *sensu lato*, *A. mayri* Forel, *A. collare* Emery) (Wheeler 1910; Weber 1941, 1945, 1972). "*Apterostigma wasmanii* [=*auriculatum*] constructs the larger nests [among *Apterostigma* spp.], and it is only in the garden of this species that the mycelium produces structures analogous to the kohlrabi heads and clusters (=gongylidia) of *Acromyrmex*" (these structures are produced only by lepiotaceous symbionts). The Pterulaceae-cultivating species of *Apterostigma* live "…in feeble colonies of only twelve to twenty individuals…." (Wheeler 1910). This colony size character in general contradicts the observed trend in the attines and, if the lepiotaceous cultivars are the ancestral ones, it would indicate a dramatic reversal in this character. The selective pressures towards such a change are unclear.

In biogeography, the nuclei of high intra-taxonomic diversity are usually considered the centers of origins of taxa (Vavilov 1951, in Schery 1972). In this respect, *Apterostigma* can be interpreted as one such diversity-rich group. Although the fungal symbiont is not an ant character *per se*, its diversity presumably reflects the ant genus' genetic diversity.

Another possible primitive trait in *Apterostigma* is the lack of the cordate head that is characteristic of other attine genera. In these, most of the head is filled with the powerful adductor muscles of the mandibles used for effective cutting/processing of substrate for

fungal cultivation. These traits are particularly notable in the higher attines. Substrate fragmentation and cutting in *Apterostigma* is possibly the least developed among the attines (Wheeler 1910; Weber 1972). The evolutionary direction of such characters in *Apterostigma* is not clear, and it may possibly be more parsimonious to consider the cordate head and mandibular muscle development to be ancestral conditions for the group. However, the lack of a solid candidate for a sister group of the attines limits the clarification of this character status. The apparently "degenerate" condition of the comparatively small, non-cordate head character in *Apterostigma* and its evolutionary direction (as well as the selective pressures leading to this change) should be discussed

6.10 THE ANCESTRAL ATTINE PROFILE; PROCESSES OF FUNGAL SYMBIONT CONSERVATION AND TRANSMISSION

Ants have extended the parental care activities, so prevalent in the Hymenoptera, across generations and reciprocally towards the mother queen. Different ant groups have been particularly pre-adapted by largely unknown mechanisms to exploit but also to protect limited renewable resources, towards their sustainable utilization (e.g. aphids; coccids, mealybugs; other ants' pupae, in the facultative and obligate ant slave-makers; and the fungi of the attines) (Wheeler 1910, Hölldobler and Wilson 1990, Schultz and McGlynn 2000). Diverse ant groups have separately developed complicated mechanisms to maintain and exploit these resources in the present in a manner that also conserves them for future use. These presumably general, polyphyletic (present in diverse ant lineages), and still obscure mechanisms conducive to sustained exploitation of resources rather than to their immediate and destructive consumption have obviously been essential to the emergence of the attine-fungus symbiosis.

I propose that the ancestral attine was arboreal, small (1-3 mm), with little queen polymorphism, and had colonies of a few dozen workers. Polygynous colonies of this ant nested in preformed cavities in wood, including those of bark beetles, like leptothoracine ants do (Hölldobler and Wilson 1990) and adopted mated founder queens after mating

flights. These colonies probably reproduced by budding. These traits facilitated the development of mycophagy and subsequently eased fungal transmission over time.

The recognition and adoption mechanisms between unrelated queens, workers and fungus outlined below are described within the framework defined by the proposed interaction with the preexisting beetle-fungus symbiosis. However, the adoption processes and mechanisms described would also be relevant for other hypotheses attempting to explain the origin of the attine-fungus symbiosis. Therefore, much of my analysis of early attine-fungus association can be interpreted broadly and is not wholly restricted to the hypothetical evolutionary route that I propose. These mechanisms are not mutually exclusive within ants. Indeed, they are known to operate simultaneously in both attine and non-attine ant genera.

I propose that relatively non-stringent, lenient queen-worker recognition mechanisms and the bypassing of strict monogynous vertical colony foundation allowed the continuation of the incipient attine ant-fungus symbiosis. These "permissive" interactions allowed queen adoption in the early symbiotic colonies. Empirical evidence supports these potential relaxed interactions. Even in the most specialized and derived genus of attine ants, *Atta*, the "adoption" and stable association of unrelated queens, workers, and fungus symbionts is possible in small laboratory colonies (Sánchez-Peña, unpublished observations). Regarding symbiont consumption and adoption, even *Atta* is a facultatively "broad" mycophagist. *Atta* colonies will feed on fungi from other attine genera, like *Trachymyrmex* (Weber 1972); they will cultivate and develop colonies using these alien cultivars (Sánchez-Peña, unpublished observations). In rRNA phylogeny studies (Sogin and Hinkle 1996) the asexual symbionts of *Atta* and *Trachymyrmex* were considered to belong in different genera. In the primitive, lower attines, horizontal adoption of distantly related fungal symbionts has been widespread (Mueller et al. 1998). They proposed disturbance and mixing of nests as one mechanism of symbiont change.

The critical process of cultivar maintenance and transmission could have developed if the incipient fungus-feeding Paleoattines were facultatively and/or secondarily polygynous.

In subsequent colony budding, adopted queens from these secondarily polygynous colonies could leave the adoptive nest along with some workers, brood and the proto-symbiotic fungus as food. Alternatively, adopted, newly mated queens could simply outlive the adoptive queen(s) and take over the "orphaned" colony and its protosymbiotic fungus. Cooperative colony founding among attine ant queens has been observed in *Atta texana* (Buckley), *Acromymex echiniator* (Forel), *Acromymex versicolor* Pergande, and *Apterostigma* spp. (Weber 1945, 1972, Mintzer and Vinson 1985, Rissing et al. 1989, Bekkevold et al. 1999). Newly mated queen adoption occurs in several ant subfamilies including the Myrmicinae (Vargo and Porter 1989; Hölldobler and Wilson 1990).

Additionally, many attine colonies have separated cavities, each containing fungus and brood. Under stress, isolated cavities could conceivably adopt a queen. Weber (1972) describes a possible case of queen adoption in *Cyphomyrmex*. I consider queen adoption to be a method to bypass vertical fungal transmission between nests over time, and, therefore, favorable for symbiosis continuation during its early evolutionary stages.

Carry-over of natal or adoptive nest elements by departing ant queens does occur. In some ants, foundresses take members of their original colony with them, to help in new colony establishment, as it occurs in colony budding in the myrmicine *Solenopsis invicta* Buren (Vargo and Porter 1989). Prior to colony foundation, queens of several species of the formicine *Acropyga* take along starter cultures of their seemingly obligate symbiotic mealybugs (Hemiptera: Pseudococcidae) from which a new generation of mealybugs will be started in the newly founded ant colony (Hölldobler and Wilson 1990, Schultz and McGlynn 2000, LaPolla et al. 2002). In *Acropyga exsanguis* (Wheeler), both colony founding by multiple queens and adoption of young queens by established colonies have been reported (Bünzli 1935); these mechanisms were probably significant for the initial continuity of this ant-homopteran symbiosis.

In the attines, several behavioral responses are the same for fungus and brood. Worker-protective behaviors towards the fungus and brood are similar and notorious. The ants retrieve and transport cultivar fragments and pile them up with the brood. Upon nest

destruction or perturbance, both cultivar and brood are carried to safety and to new nesting sites (Weber 1972; Viana et al 2001; Sánchez-Peña, unpublished observations). In retrieving and transport tests with *Acromyrmex subterraneus* Forel workers, the fungus was treated as brood (Viana et al. 2001). *Acropyga epedana* Snelling also mixes its symbiotic mealybugs with the brood (LaPolla et al. 2002).

Vertical transmission of the symbiont in the mouth (infrabuccal pocket) of founding queens developed from the ants' external transport of the fungus by holding it with the mandibles or mouthparts. The founding queens of many attines (certainly the higher genera: *Sericomyrmex, Trachymyrmex, Acromyrmex* and *Atta*) transport the inoculum for initial fungus garden establishment in the infrabuccal pocket, a post-oral widening of the digestive tube. The queens of these highly derived attine species usually engage in more or less extensive digging before they eject the fungus and start tending it. I propose that founder queens of the ancestral attines carried a piece of fungus as inoculum externally (probably holding it with the mandibles' base). Further, that this habit, found at least in the higher attines, of carrying such fungal inoculum in the infrabuccal pocket originated from selective pressures to protect the fungus better during mating flights or to free the mandibles for digging. To what extent this pocket is used as a transportation device by ants in general is not known.

That the queens of the early attines could have carried the fungus externally is shown by many lower attines (e.g., in *Apterostigma*, *Cypomyrmex* and even some *Acromyrmex* species) that nest in preformed cavities in soil, wood, rolled leaves in litter, between bark and wood of trees, or in litter accumulations (Weber 1972). This nesting habit is thus primitive and the ancestral founder queens did not need to dig into soil. This, in turn, would have avoided conflict between carrying fungus in the mandibles and using the mandibles for digging, allowing for the transportation of the fungal symbiont in the mandibles prior to the evolution of temporary infrabuccal storage..

6.11 ORIGIN OF AMBROSIA BEETLES-FUNGAL MUTUALISM

6.11.1 Ancestral Beetle-Fungus Interactions in Wood.

The adventitious fungal flora naturally colonizing beetle galleries in wood would have exposed these originally wood-feeding insects, to a natural, large-scale experiment on mycophagy, in which nutritional adequacy of fungi drastically influenced beetle survival (Batra 1966). Beetle-antagonistic bluestain fungi (Ophiostomatales) colonize trees and individual branches of bark beetle-infested conifers. These trees and branches show distorted beetle galleries and aborted larval development (Ayres et al 2000; Klepzig et al. 2001a, 2001b; Sánchez-Peña., unpublished observations). Beetle survival obviously has a direct effect upon subsequent transport of fungi to new plant hosts by beetles. This transport was initially fortuitous external phoresy on the adult beetles' integument. The beetle cohorts that chanced to feed upon more adequate (or less antagonistic) fungal strains coexisting with them in wood were better developed (larger, more vigorous or numerous, etc.) and transmitted these fungi more efficiently. These "beneficial" fungi probably also provided varying degrees of antibiosis against beetle-antagonistic fungi colonizing opportunistically the wood the beetles were developing upon. In fact it has been shown that the beetle-symbiotic *Entomocorticium* can prevent wood and gallery colonization by beetle-antagonistic *Ophiostoma* species (Klepzig et al 2001 a, 2001b).

6.12 CONCLUDING REMARKS; SUMMARY

I propose the following hypothesis regarding the origin of attine and beetle (Platypodinae +Scolytinae) fungus mutualisms.

1) The ancestral Scolytoid (Platypodinae + Scolytinae) beetles evolved towards a xylophagous and, subsequently, a mycophagous diet. In this process these beetles "adopted", fed upon, and then became intimately associated with, specific fungal guilds developing fortuitously in their nest galleries. These fungi may have initially been competitors, but those that were less antagonistic (or perhaps favorable) to beetle development became even more intimately associated with the beetles. In addition, fungi

could have provided some antibiotic properties against more markedly beetle-antagonistic fungi co-habiting beetle galleries. Phoresy of the fungi on the beetles developed and beetles became vectors for fungal cultures when they dispersed to found new gallery systems.

2) The ancestors of extant attines first developed the mycophagous habit from feeding (either predaciously or opportunistically, in abandoned galleries) on fungi previously domesticated by, and associated as food items with, ambrosia beetles' galleries in wood. These ant ancestors had a mixed diet of beetle-fungus and beetle brood, but became more markedly mycophagous. Fungi were probably a more abundant or long-lasting resource than brood in the beetle galleries that they moved into, simply due to their indeterminate, continuous growth.

4) The transitional, facultative mycophagous proto-attines disappeared. The causes are unclear, but if the ant association with fungi developed from opportunistic exploitation of fungi of Siricoid wasps instead of beetles, the extinction of these wasps could explain the disappearance of host-specific, associated mycophagous ants analogous to *Megalomyrmex*.

5) Adaptations evolved that enabled the maintenance and then development of the ants' own fungus gardens. Colony budding and/or founding queen adoption allowed the maintenance and continuation of the incipient association with specific fungal taxa. The wood-rotting cultivars (such as those of *Apterostigma*) were discovered and adopted and they were perhaps best-suited symbionts..

6) Vertical transmission of the symbiont in the infrabuccal pocket of founding queens developed from external (mandibular, maxillary, etc.) transport of the fungus.

7) The development of broad mycophagy, and overcoming the possible limitations and challenges (toxins, nutrients) concomitant with it, allowed these ants to sample other fungi from their environment. "Testing" of environmental isolates of fungi allowed the ancestral attines to domesticate strains more apt as symbionts and eventually, even more

apt cultivars (Lepiotaceae) were discovered (in very decayed stumps or in litter?), and these, too, were adopted; they allowed the phenomenal adaptive radiation of Lepiotaceae-cultivating attines (Weber 1972, Garcia et al. 2002).

8) The cultivation of pterulaceous, wood-colonizing Basidiomycota by the very primitive, basal attine *Apterostigma* (Moncalvo et al. 2000, Munkacsi and McLauglin 2001, Munkacsi et al. 2004) is interpreted as possibly more basal, not as derived as the cultivation of lepiotaceous symbionts..

6.13 PREDICTIONS

The Attini constitute a clearly monophyletic group (Weber 1972, Wetterer et al. 1998, Schultz 2000). The fact that no facultatively mycophagous or facultative fungal cultivator exists in this group of ants indicates that these facultative stages were evolutionarily ephemeral, and were pervasively replaced by obligate fungicultural habits (Chapela et al. 1994, Mueller et al 1998). It is possible also that the closest relatives of the attines are undiscovered facultative fungivores or facultative gardeners (Mueller et al 2001).

My purpose is to encourage hypothesis testing within the theoretical framework proposed herein. It is possible that in performing empirical (especially behavioral) investigations of attine mycophagy and fungus cultivation, one might be analyzing an ancient and highly derived system well removed from the postulated ancestral state of flexible mycophagy, therefore obscuring evolutionary interpretations. Nonetheless, I propose the following testable predictions, in which "beetles" refers to fungus-symbiotic Scolytinae + Platypodinae.

1) Attines (more probably the lower, primitive attines) will feed on the basidiomycetous (less probably on the ascomycetous) symbionts of beetles. If they do, it will probably be without strong or acute deletereous effects (toxicity) on the ants. Additionally, primitive attines (*Apterostigma*, *Mycocepurus*, *Myrmycocrypta*) may be found to feed on beetle fungi in the field.

2) Ants will be found in the sister taxon of attines, as well as in other ant taxa, that will feed on beetle larvae (probably) and on beetle larvae + fungus (less probably?) in the field in South America. This sister taxon is probably the omnivorous, not-known-to-be-fungivorous clade *Blepharidatta/Wasmannia*. However, the "sister taxon" has not been identified with certainty, since both molecular and morphological data are unclear.

3) Non-agricultural mycophagous ants exist in the Neotropics associated with galleries of siricoid wasps.

4). In the South American tropics, beetle Basidiomycota can be found, that are more closely related to known attine fungi (wood Pterulaceae and Tricholomataceae) than those currently known.

5) Conversely, attines will be found cultivating fungi more closely related to the lignicolous "Aphyllophorales" (a polyphyletic basidiomycetous group that includes the beetle fungi). It is much less likely that ascomycetous ophiostomatoid fungi will be found as attine symbionts.

6). Workers of primitive attine ants (*Apterostigma?)* will adopt and attempt to cultivate some of the fungal symbionts of beetles from wood.

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Vita

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